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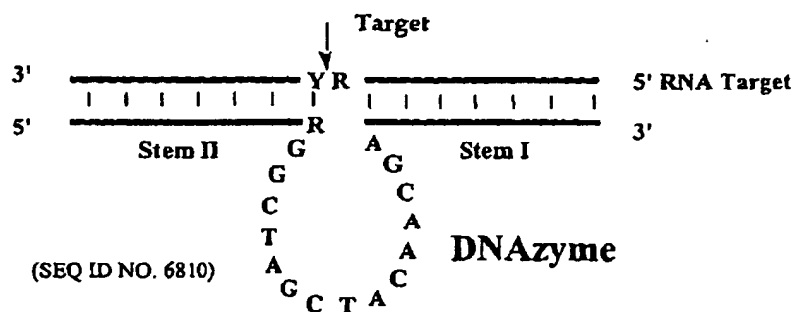
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(54) Title: **NUCLEIC ACID TREATMENT OF DISEASES OR CONDITIONS RELATED TO LEVELS OF RAS, HER2 AND HIV*****DNAzyme Motif*****Legend**
 Y = U or C
 R = A or G

(57) Abstract: The present invention relates to nucleic acid molecules, including enzymatic nucleic acid molecules, such as DNAzymes (e.g. DNA enzymes, catalytic DNA), siRNA, aptamers, and antisense that modulate the expression of Ras genes such as K-Ras, H-Ras, and/or N-Ras, HIV genes such as HIV-1, and HER2 genes.

WO 02/097114 A2

WO 02/097114 A2



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DESCRIPTION

NUCLEIC ACID TREATMENT OF DISEASES OR CONDITIONS RELATED TO LEVELS OF RAS, HER2 AND HIV

This patent application claims priority from McSwiggen USSN 60/294,140, filed May 29, 2001, entitled "Enzymatic Nucleic Acid Treatment of Diseases or Conditions Related To Levels of HIV," McSwiggen USSN 60/296,249 filed June 6, 2001, entitled "Enzymatic Nucleic Acid Treatment of Diseases or Conditions Related to Levels of HER2," and McSwiggen USSN 60/318,471, filed September 10, 2001, entitled "Enzymatic Nucleic Acid Treatment of diseases or Conditions Related to Levels of RAS." Each of these applications is hereby incorporated by reference herein in its entirety including the drawings and tables.

Technical Field Of The Invention

The present invention relates to novel nucleic acid compounds and methods for the treatment or diagnosis of diseases or conditions related to levels of Ras gene expression, such as K-Ras, H-Ras, and/or N-Ras expression, HIV infection such as HIV-1, and HER2 gene expression.

Background Of The Invention

Transformation is a cumulative process whereby normal control of cell growth and differentiation is interrupted, usually through the accumulation of mutations affecting the expression of genes that regulate cell growth and differentiation.

The platelet derived growth factor (PDGF) system has served as a prototype for identification of substrates of the receptor tyrosine kinases. Certain enzymes become activated by the PDGF receptor kinase, including phospholipase C and phosphatidylinositol 3' kinase, Ras guanosine triphosphate (GTPase) activating protein (GAP) and src-like tyrosine kinases. GAP regulates the function of the Ras protein by stimulating the GTPase activity of the 21 kD Ras protein. Barbacid, 56 Ann. Rev. Biochem. 779, 1987. Microinjection of oncogenically activated Ras into NIH 3T3 cells has been shown to induce DNA synthesis. Mutations that cause oncogenic activation of Ras lead to accumulation of Ras bound to GTP, the active form of the molecule. These mutations block the ability of GAP to convert Ras to the inactive form. Mutations that impair the interactions of Ras with GAP also block the biological function of Ras.

While a number of Ras alleles exist (N-Ras, K-Ras, H-Ras) which have been implicated in carcinogenesis, the type most often associated with colon and pancreatic carcinomas is K-Ras. Enzymatic nucleic acid molecules which are targeted to certain regions of the K-Ras allelic mRNAs may also prove inhibitory to the function of the other allelic mRNAs of the N-Ras and H-Ras genes.

Scanlon, International PCT Publication Nos. WO 91/18625, WO 91/18624, and WO 91/18913 describes a ribozyme effective to cleave oncogene RNA from the H-Ras gene. This ribozyme is said to inhibit H-ras expression in response to exogenous stimuli. Reddy WO92/00080 describes the use of ribozymes as therapeutic agents for leukemias, such as chronic myelogenous leukemia (CML) by targeting specific portions of the BCR-ABL gene transcript.

Thompson *et al.*, International PCT publication No. WO 99/54459, describe nucleic acid molecules that modulate gene expression, including Ras gene expression.

Zhang *et al.*, 2000, *Gene Ther.*, 7, 2041; Takunaga *et al.*, 2000, *Br. J. Cancer.*, 83, 833; Zhang *et al.*, 2000, *Mol. Biotechnol.*, 15, 39; Irie *et al.*, 2000, *Mol. Urol.* 4, 61; Kijima and Scanlon, 2000, *Mol. Biotechnol.*, 14, 59; Funato *et al.*, 2000, *Cancer Gene Ther.*, 7, 495; Tsuchida *et al.*, 2000, *Cancer Gene Ther.*, 7, 373; Zhang *et al.*, 2000, *Methods Mol. Med.*, 35, 261; Irie *et al.*, 1999, *Antisense Nucleic Acid Drug Dev.*, 9, 341; Giannini *et al.*, 1999, *Nucleic Acids Res.*, 27, 2737; Fang *et al.*, 1999, *J. Med. Coll. PLA*, 14, 25; Tong *et al.*, 1998, *Methods Mol. Med.*, 11, 209; Ohkawa and Kashani-Sabet, 1998, *Methods Mol. Med.*, 11, 153; Scherr *et al.*, 1999, *Gene Ther.*, 6, 152; Tsuchida *et al.*, 1998, *Biochem. Biophys. Res. Commun.*, 252, 368; Scherr *et al.*, 1998, *Gene Ther.*, 5, 1227; Uhlmann *et al.*, European Patent Application EP 808898; Scherr *et al.*, 1997, *J. Biol. Chem.*, 272, 14304; Chang *et al.*, 1997, *J. Cancer Res. Clin. Oncol.*, 123, 91; Ohta *et al.*, 1996, *Nucleic Acids Res.*, 24, 938; Ohta *et al.*, 1994, *Ann. N.Y. Acad. Sci.*, 716, 242; and Funato *et al.*, 1994, *Biochem. Pharmacol.*, 48, 1471 all describe specific ribozymes targeting certain K-Ras, H-Ras, or N-Ras RNA sequences.

Todd, International PCT Publication Nos. WO 01/49877, WO 99/50452, and WO 99/45146 describes specific DNazymes targeting K-Ras for diagnostic applications.

Acquired immunodeficiency syndrome (AIDS) is thought to be caused by infection with the human immunodeficiency virus, for example HIV-1. Draper *et al.*, U.S. Patent Nos. 6,159,692, 5,972,704, 5,693,535, and International PCT Publication Nos. WO WO 93/23569,

WO 95/04818, describe enzymatic nucleic acid molecules targeting HIV. Todd *et al.*, International PCT Publication No. WO 99/50452, describe methods for using specific DNAzyme motifs for detecting the presence of certain HIV RNAs. Sriram and Banerjee, 2000, *Biochem J.*, 352, 667-673, describe specific RNA cleaving DNA enzymes targeting
5 HIV-1. Zhang *et al.*, 1999, *FEBS Lett.*, 458, 151-156, describe specific RNA cleaving DNA enzymes used in the inhibition of HIV-1 infection.

HER2 (also known as neu, erbB2 and c-erbB2) is an oncogene that encodes a 185-kDa transmembrane tyrosine kinase receptor. HER2 is a member of the epidermal growth factor receptor (EGFR) family and shares partial homology with other family members. In normal
10 adult tissues HER2 expression is low. However, HER2 is overexpressed in at least 25-30% of breast (McGuire, H.C. and Greene, M.I. (1989) The *neu* (c-erbB-2) oncogene. *Semin. Oncol.* 16: 148-155) and ovarian cancers (Berchuck, A. Kamel, A., Whitaker, R. *et al.* (1990)). Overexpression of her-2/neu is associated with poor survival in advanced epithelial ovarian cancer. *Cancer Research* 50: 4087-4091). Furthermore, overexpression of HER2 in
15 malignant breast tumors has been correlated with increased metastasis, chemoresistance and poor survival rates (Slamon *et al.*, 1987 *Science* 235: 177-182). Because HER2 expression is high in aggressive human breast and ovarian cancers, but low in normal adult tissues, it is an attractive target for enzymatic nucleic acid-mediated therapy. McSwiggen *et al.*, International PCT Publication No. WO 01/16312 and Beigelman *et al.*, International PCT Publication No.
20 WO 99/55857 describe enzymatic nucleic acid molecules targeting HER2. Thompson and Draper, US Patent No. 5,599,704, describes enzymatic nucleic acid molecules targeting HER2 (erbB2/neu) gene expression.

Summary Of The Invention

The present invention features nucleic acid molecules, including, for example, antisense
25 oligonucleotides, siRNA, aptamers, decoys and enzymatic nucleic acid molecules such as DNAzyme enzymatic nucleic acid molecules, which modulate expression of nucleic acid molecules encoding Ras oncogenes, such as K-Ras, H-Ras, and N-Ras. In one embodiment, the invention features an enzymatic nucleic acid molecule comprising a sequence selected from the group consisting of SEQ ID NOs: 2329-4655.

In another embodiment, the invention features an enzymatic nucleic acid molecule comprising at least one binding arm having a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs: 1-2328.

5 In another embodiment, the invention features a siRNA molecule having complementarity to a sequence selected from the group consisting of SEQ ID NOs: 1-2328.

In another embodiment, the invention features an antisense molecule having complementarity to a sequence selected from the group consisting of SEQ ID NOs: 1-2328.

In another aspect of the invention, the nucleic acid of the invention is adapted to treat cancer.

10 In one embodiment, the enzymatic nucleic acid molecule of the invention has an endonuclease activity to cleave RNA having a K-Ras sequence.

In another embodiment, the enzymatic nucleic acid molecule of the invention has an endonuclease activity to cleave RNA having an H-Ras sequence.

15 In another embodiment, the enzymatic nucleic acid molecule of the invention has an endonuclease activity to cleave RNA having an N-Ras sequence.

In one embodiment, the siRNA molecule of the invention has RNA interference activity to K-Ras expression.

In another embodiment, the siRNA molecule of the invention has RNA interference activity to H-Ras expression.

20 In another embodiment, the siRNA molecule of the invention has RNA interference activity to N-Ras expression.

In one embodiment, a siRNA molecule of the invention comprises a double stranded RNA wherein one strand of the RNA is complementary to the RNA of K-Ras, H-Ras, and/or N-Ras gene. In another embodiment, a siRNA molecule of the invention comprises a double
25 stranded RNA wherein one strand of the RNA comprises a portion of a sequence of RNA of K-Ras, H-Ras, and/or N-Ras gene sequence. In yet another embodiment, a siRNA molecule of the invention comprises a double stranded RNA wherein both strands of RNA are connected by a non-nucleotide linker. Alternately, a siRNA molecule of the invention comprises a double stranded RNA wherein both strands of RNA are connected by a
30 nucleotide linker, such as a loop or stem loop structure.

In one embodiment, a single strand component of a siRNA molecule of the invention is from about 14 to about 50 nucleotides in length. In another embodiment, a single strand component of a siRNA molecule of the invention is about 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 nucleotides in length. In yet another embodiment, a single strand component of a siRNA molecule of the invention is about 23 nucleotides in length. In one embodiment, a siRNA molecule of the invention is from about 28 to about 56 nucleotides in length. In another embodiment, a siRNA molecule of the invention is about 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, or 52 nucleotides in length. In yet another embodiment, a siRNA molecule of the invention is about 46 nucleotides in length.

In one embodiment, the DNAzyme molecule of the invention is in a "10-23" configuration (see for example Santoro *et al.*, 1997, *PNAS*, 94, 4262 and Joyce *et al.*, US 5,807,718). In another embodiment, the DNAzyme comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs: 1-2328. In yet another embodiment, the DNAzyme comprises a sequence selected from the group consisting of SEQ ID NOs: 2329-4655.

In another embodiment, the nucleic acid molecule of the invention comprises between 12 and 100 bases complementary to a nucleic acid molecule having a K-Ras sequence. In yet another embodiment, the enzymatic nucleic acid comprises between 14 and 24 bases complementary to a nucleic acid molecule having a K-Ras sequence.

In another embodiment, the nucleic acid molecule of the invention comprises between 12 and 100 bases complementary to a nucleic acid molecule having an H-Ras sequence. In yet another embodiment, the nucleic acid molecule of the invention comprises between 14 and 24 bases complementary to a nucleic acid molecule having an H-Ras sequence.

In another embodiment, the nucleic acid molecule of the invention comprises between 12 and 100 bases complementary to a nucleic acid molecule having an N-Ras sequence. In yet another embodiment, the nucleic acid molecule of the invention comprises between 14 and 24 bases complementary to a nucleic acid molecule having an N-Ras sequence.

In yet another embodiment, the nucleic acid molecule of the invention is chemically synthesized. The nucleic acid molecule can comprise at least one 2'-sugar modification, at least one nucleic acid base modification, and/or at least one phosphate backbone modification.

In one embodiment, the invention features a mammalian cell comprising the nucleic acid molecule of the invention. In another embodiment, the mammalian cell of the invention is a human cell.

5 In another embodiment, the invention features a method of modulating K-Ras activity in a cell, comprising contacting the cell with the nucleic acid molecule of the invention, under conditions suitable for the modulation of K-Ras activity.

In another embodiment, the invention features a method of modulating H-Ras activity in a cell, comprising contacting the cell with the nucleic acid molecule of the invention, under conditions suitable for the modulation of H-Ras activity.

10 In another embodiment, the invention features a method of modulating N-Ras activity in a cell, comprising contacting the cell with the nucleic acid molecule of the invention, under conditions suitable for the modulation of N-Ras activity.

15 In another embodiment, the invention features a method of treatment of a subject having a condition associated with the level of K-Ras, comprising contacting cells of the subject with the nucleic acid molecule of the invention, under conditions suitable for the treatment.

20 In another embodiment, the invention features a method of treatment of a subject having a condition associated with the level of H-Ras, comprising contacting cells of the subject with the nucleic acid molecule of the invention, under conditions suitable for the treatment.

In another embodiment, the invention features a method of treatment of a subject having a condition associated with the level of N-Ras, comprising contacting cells of the subject with the nucleic acid molecule of the invention, under conditions suitable for the treatment.

25 In one embodiment, a method of treatment of the invention further comprises the use of one or more drug therapies under conditions suitable for the treatment.

30 In another embodiment, the invention features a method of cleaving RNA having a K-Ras sequence comprising contacting the K-Ras RNA with the enzymatic nucleic acid molecule of the invention under conditions suitable for the cleavage, for example, where the cleavage is carried out in the presence of a divalent cation, such as Mg²⁺.

In another embodiment, the invention features a method of cleaving RNA having a H-Ras sequence comprising contacting the H-Ras RNA with the enzymatic nucleic acid molecule of the invention under conditions suitable for the cleavage, for example, where the cleavage is carried out in the presence of a divalent cation, such as Mg^{2+} .

- 5 In another embodiment, the invention features a method of cleaving RNA having an N-Ras sequence comprising contacting the N-Ras RNA with the enzymatic nucleic acid molecule of the invention under conditions suitable for the cleavage, for example, where the cleavage is carried out in the presence of a divalent cation, such as Mg^{2+} .

- 10 In one embodiment, the nucleic acid molecule of the invention comprises a cap structure, for example, a 3',3'-linked or 5',5'-linked deoxyabasic ribose derivative, wherein the cap structure is at the 5'-end, 3'-end, or both the 5'-end and the 3'-end of the nucleic acid molecule.

- 15 In another embodiment, the invention features an expression vector comprising a nucleic acid sequence encoding at least one nucleic acid molecule of the invention in a manner that allows expression of the nucleic acid molecule. For example, the invention features an expression vector comprising a nucleic acid encoding a DNAzyme in a manner that allows expression of the DNAzyme.

In yet another embodiment, the invention features a mammalian cell, for example a human cell, comprising an expression vector of the invention.

- 20 In another embodiment, the expression vector of the invention further comprises a sequence for a nucleic acid molecule complementary to an RNA having K-Ras sequence.

In another embodiment, the expression vector of the invention further comprises a sequence for a nucleic acid molecule complementary to an RNA having H-Ras sequence.

- 25 In another embodiment, the expression vector of the invention further comprises a sequence for a nucleic acid molecule complementary to an RNA having N-Ras sequence.

- 30 In one embodiment, an expression vector of the invention comprises a nucleic acid sequence encoding two or more nucleic acid molecules of the invention, which can be the same or different. In another embodiment, an expression vector of the invention further comprises a sequence encoding an antisense nucleic acid molecule complementary to an RNA having a K-Ras, H-Ras or N-Ras sequence.

In another embodiment, the invention features a method for treating cancer, for example colorectal cancer, bladder cancer, lung cancer, pancreatic cancer, breast cancer, or prostate cancer, comprising administering to a subject a nucleic acid molecule of the invention under conditions suitable for the treatment. A method of treatment of cancer of the invention can further comprise administering to a patient one or more other therapies, for example, monoclonal antibody therapy, such as Herceptin (trastuzumab); chemotherapy, such as paclitaxel (Taxol), docetaxel, cisplatin, methotrexate, cyclophosphamide, doxorubin, fluorouracil carboplatin, Leucovorin, Irinotecan (CAMPTOSAR® or CPT-11 or Camptothecin-11 or Campto), Carboplatin, edatrexate, gemcitabine, or vinorelbine; radiation therapy, or analgesic therapy and/or any combination thereof.

In another embodiment, the invention features a composition comprising a nucleic acid molecule of the invention in a pharmaceutically acceptable carrier.

In one embodiment, the invention features a method of administering to a cell, for example a mammalian cell or human cell, the nucleic acid molecule of the invention comprising contacting the cell with the nucleic acid molecule under conditions suitable for administration. The method of administration can be in the presence of a delivery reagent, for example a lipid, cationic lipid, phospholipid, or liposome.

The present invention features an enzymatic nucleic acid molecule which modulates expression of a nucleic acid molecule encoding a human immunodeficiency virus (HIV), for example HIV-1, HIV-2, and related viruses such as FIV-1 and SIV-1, or a HIV gene, for example LTR, nef, vif, tat, or rev, wherein the enzymatic nucleic acid molecule comprises a DNAzyme configuration.

The invention also features an enzymatic nucleic acid molecule which modulates expression of a nucleic acid molecule encoding HIV or a component of HIV such as net, vif, tat, or rev, wherein the enzymatic nucleic acid molecule is in a Inozyme, G-cleaver, Zinzyme, DNAzyme or Amberzyme configuration.

The present invention also features a siRNA molecule which modulates expression of a nucleic acid molecule encoding a human immunodeficiency virus (HIV), for example HIV-1, HIV-2, and related viruses such as FIV-1 and SIV-1, or a HIV gene, for example LTR, nef, vif, tat, or rev.

The present invention features an enzymatic nucleic acid molecule comprising a sequence selected from the group consisting of SEQ ID NOs. 6727-6799. The invention also features an enzymatic nucleic acid molecule comprising at least one binding arm wherein one

or more of said binding arms comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6642-6726. In addition, the present invention features a siRNA nucleic acid molecule comprising sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 1-76 and 140-148.

- 5 In another embodiment, the siRNA molecule of the invention has RNA interference activity to HIV-1 expression and/or replication.

In one embodiment, a siRNA molecule of the invention comprises a double stranded RNA wherein one strand of the RNA is complementary to the RNA of HIV-1 genome or genes. In another embodiment, a siRNA molecule of the invention comprises a double
10 stranded RNA wherein one strand of the RNA comprises a portion of a sequence of HIV-1 genome or gene sequence. In yet another embodiment, a siRNA molecule of the invention comprises a double stranded RNA wherein both strands of RNA are connected by a non-nucleotide linker. Alternately, a siRNA molecule of the invention comprises a double
15 stranded RNA wherein both strands of RNA are connected by a nucleotide linker, such as a loop or stem loop structure.

In one embodiment, a single strand component of a siRNA molecule of the invention is from about 14 to about 50 nucleotides in length. In another embodiment, a single strand component of a siRNA molecule of the invention is about 14, 15, 16, 17, 18, 19, 20, 21, 22,
20 23, 24, 25, 26, 27, or 28 nucleotides in length. In yet another embodiment, a single strand component of a siRNA molecule of the invention is about 23 nucleotides in length. In one embodiment, a siRNA molecule of the invention is from about 28 to about 56 nucleotides in length. In another embodiment, a siRNA molecule of the invention is about 40, 41, 42, 43,
25 44, 45, 46, 47, 48, 49, 50, 51, or 52 nucleotides in length. In yet another embodiment, a siRNA molecule of the invention is about 46 nucleotides in length.

- 25 In one embodiment, a nucleic acid molecule of the invention is adapted to treat HIV infection or acquired immunodeficiency syndrome (AIDS).

In another embodiment, the enzymatic nucleic acid molecule of the invention has an endonuclease activity to cleave RNA having HIV sequence.

- 30 In yet another embodiment, the enzymatic nucleic acid molecule of the invention is in an Inozyme, Zinzyme, G-cleaver, Amberzyme, DNAzyme or Hammerhead configuration.

In another embodiment, the Inozyme of the invention comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6648-6655, or comprises a sequence selected from the group consisting of SEQ ID NOs. 6733-6740.

5 In another embodiment, the Zinzyme of the invention comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6656-6663 and 6723-6726, or comprises a sequence selected from the group consisting of SEQ ID NOs 6741-6748 and 6795-6799.

10 In another embodiment, the Amberzyme of the invention comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6656-6688, or comprises a sequence selected from the group consisting of SEQ ID NOs. 6762-6789.

In another embodiment, the DNAzyme of the invention comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6656-6668 and 6718-6722, or comprises a sequence selected from the group consisting of SEQ ID NOs. 6749-6761 and 6790-6794.

15 In another embodiment, the Hammerhead of the invention comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6642-6647, or comprises a sequence selected from the group consisting of SEQ ID NOs 6727-6732.

In one embodiment, a nucleic acid molecule of the invention comprises between 12 and 100 bases complementary to a RNA sequence encoding HIV genome, RNA, and/or proteins.

20 In another embodiment, a nucleic acid molecule of the invention comprises between 14 and 24 bases complementary to a RNA sequence encoding HIV genome, RNA, and/or proteins.

25 In yet another embodiment, a nucleic acid molecule of the invention is chemically synthesized. A nucleic acid molecule of the invention can comprise at least one 2'-sugar modification, at least one nucleic acid base modification, and/or at least one phosphate backbone modification.

The present invention features a mammalian cell including a nucleic acid molecule of the invention. In one embodiment, the mammalian cell of the invention is a human cell.

30 The invention features a method of reducing HIV activity in a cell, comprising contacting the cell with a nucleic acid molecule of the invention, under conditions suitable for the reduction of HIV activity.

The invention also features a method of treating a subject having a condition associated with the level of HIV, comprising contacting cells of the subject with a nucleic acid molecule of the invention, under conditions suitable for the treatment.

5 In one embodiment, methods of treatment contemplated by the invention comprise the use of one or more drug therapies under conditions suitable for the treatment.

The invention features a method of cleaving RNA comprising a HIV nucleic acid sequence comprising contacting an enzymatic nucleic acid molecule of the invention with the RNA under conditions suitable for the cleavage. In one embodiment, the cleavage contemplated by the invention is carried out in the presence of a divalent cation, for example
10 Mg^{2+} .

The present invention features a method for treatment of acquired immunodeficiency syndrome (AIDS) or an AIDS related condition, for example Kaposi's sarcoma, lymphoma, cervical cancer, squamous cell carcinoma, cardiac myopathy, rheumatic disease, or opportunistic infection, comprising administering to a subject a nucleic acid molecule of the
15 invention under conditions suitable for the treatment.

In one embodiment, nucleic acid molecule of the invention comprises at least five ribose residues, at least ten 2'-O-methyl modifications, and a 3'- end modification, for example a 3'-3' inverted abasic moiety.

20 In another embodiment, a nucleic acid molecule of the invention further comprises phosphorothioate linkages on at least three of the 5' terminal nucleotides.

In yet another embodiment, a DNAzyme of the invention comprises at least ten 2'-O-methyl modifications and a 3'-end modification, for example a 3'-3' inverted abasic moiety. In a further embodiment, the DNAzyme of the invention further comprises phosphorothioate linkages on at least three of the 5' terminal nucleotides.

25 In another embodiment, other drug therapies of the invention comprise antiviral therapy, monoclonal antibody therapy, chemotherapy, radiation therapy, analgesic therapy, or anti-inflammatory therapy.

30 In yet another embodiment, antiviral therapy of the invention comprises treatment with AZT, ddC, ddI, d4T, 3TC, Ribavirin, delvaridine, nevirapine, efavirenz, ritonavir, saquinavir, indinavir, amprenavir, nelfinavir, or lopinavir.

The invention features a composition comprising a nucleic acid molecule of the invention in a pharmaceutically acceptable carrier.

In one embodiment, the invention features a method of administering to a cell, for example a mammalian cell or human cell, an enzymatic nucleic acid molecule of the invention comprising contacting the cell with the enzymatic nucleic acid molecule under conditions suitable for the administration. The method of administration can be in the
5 presence of a delivery reagent, for example a lipid, cationic lipid, phospholipid, or liposome.

The present invention features enzymatic nucleic acid molecules which modulate expression of nucleic acid molecules encoding HER2. The present invention also features siRNA molecules which modulate the expression of nucleic acid molecules encoding HER2.

In another embodiment, the invention features a siRNA molecule having
10 complementarity to a sequence selected from the group consisting of SEQ ID NOs: 4656-5643 and 6632-6636.

In one embodiment, the invention features an enzymatic nucleic acid molecule comprising a sequence selected from the group consisting of SEQ ID NOs: 5644-6631 and 6637-6641.

15 In another embodiment, the invention features an enzymatic nucleic acid molecule comprising at least one binding arm having a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs: 4656-5643 and 6632-6636.

In yet another embodiment, a nucleic acid of the invention is adapted to treat cancer.

20 In another embodiment, an enzymatic nucleic acid molecule of the invention has an endonuclease activity to cleave RNA having HER2 sequence.

In another embodiment, the siRNA molecule of the invention has RNA interference activity to N-Ras gene expression.

In one embodiment, a siRNA molecule of the invention comprises a double stranded RNA wherein one strand of the RNA is complementary to the RNA of HER2 gene. In
25 another embodiment, a siRNA molecule of the invention comprises a double stranded RNA wherein one strand of the RNA comprises a portion of a sequence of RNA having of HER2 gene sequence. In yet another embodiment, a siRNA molecule of the invention comprises a double stranded RNA wherein both strands of RNA are connected by a non-nucleotide linker. Alternately, a siRNA molecule of the invention comprises a double stranded RNA wherein
30 both strands of RNA are connected by a nucleotide linker, such as a loop or stem loop structure.

In one embodiment, a single strand component of a siRNA molecule of the invention is from about 14 to about 50 nucleotides in length. In another embodiment, a single strand component of a siRNA molecule of the invention is about 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 nucleotides in length. In yet another embodiment, a single strand component of a siRNA molecule of the invention is about 23 nucleotides in length. In one embodiment, a siRNA molecule of the invention is from about 28 to about 56 nucleotides in length. In another embodiment, a siRNA molecule of the invention is about 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, or 52 nucleotides in length. In yet another embodiment, a siRNA molecule of the invention is about 46 nucleotides in length.

In one embodiment, a DNzyme molecule of the invention is in a "10-23" configuration. In another embodiment, a DNzyme of the invention comprises a sequence complementary to a sequence having SEQ ID NOs: 4656-5643 and 6632-6636. In yet another embodiment, a DNzyme molecule of the invention comprises a sequence having SEQ ID NOs: 5644-6631 and 6637-6641.

In another embodiment, a nucleic acid molecule of the invention comprises between 12 and 100 bases complementary to a nucleic acid molecule having HER2 sequence. In yet another embodiment, a nucleic acid molecule of the invention comprises between 14 and 24 bases complementary to a nucleic acid molecule having HER2 sequence.

In yet another embodiment, a nucleic acid molecule of the invention is chemically synthesized. A nucleic acid molecule of the invention can comprise at least one 2'-sugar modification, at least one nucleic acid base modification, and/or at least one phosphate backbone modification.

In one embodiment, the invention features a mammalian cell comprising a nucleic acid molecule of the invention. In another embodiment, the mammalian cell of the invention is a human cell.

In another embodiment, the invention features a method of reducing HER2 activity in a cell, comprising contacting the cell with the nucleic acid molecule of the invention, under conditions suitable for the reduction of HER2 activity.

In another embodiment, the invention features a method of treatment of a subject having a condition associated with the level of HER2, comprising contacting cells of the subject with the nucleic acid molecule of the invention, under conditions suitable for the treatment.

In one embodiment, a method of treatment of the invention further comprises the use of one or more drug therapies under conditions suitable for the treatment.

5 In another embodiment, the invention features a method of cleaving RNA having HER2 sequence comprising contacting an enzymatic nucleic acid molecule of the invention with the RNA under conditions suitable for the cleavage, for example, where the cleavage is carried out in the presence of a divalent cation, such as Mg^{2+} .

10 In one embodiment, a nucleic acid molecule of the invention comprises a cap structure, for example a 3',3'-linked or 5',5'-linked deoxyabasic ribose derivative, wherein the cap structure is at the 5'-end, 3'-end, or both the 5'-end and the 3'-end of the enzymatic nucleic acid molecule.

In another embodiment, the invention features an expression vector comprising a nucleic acid sequence encoding at least one nucleic acid molecule of the invention, for example a DNAzyme or siRNA molecule, in a manner that allows expression of the nucleic acid molecule.

15 In yet another embodiment, the invention features a mammalian cell, for example a human cell, comprising an expression vector of the invention.

In another embodiment, an expression vector of the invention further comprises a sequence for a nucleic acid molecule complementary to a nucleic acid molecule having HER2 sequence.

20 In one embodiment, an expression vector of the invention comprises a nucleic acid sequence encoding two or more nucleic acid molecules, which can be the same or different. In another embodiment, an expression vector of the invention further comprises a sequence encoding an antisense nucleic acid molecule complementary to a nucleic acid molecule having a HER2 sequence.

25 In another embodiment, the invention features a method for treating cancer, for example breast cancer or ovarian cancer, comprising administering to a subject a nucleic acid molecule of the invention under conditions suitable for the treatment. A method of treatment of cancer of the invention can further comprise administering to a patient one or more other therapies, for example, monoclonal antibody therapy, such as Herceptin (trastuzumab);
30 chemotherapy, such as paclitaxel (Taxol), docetaxel, cisplatin, methotrexate, cyclophosphamide, doxorubin, fluorouracil carboplatin, Leucovorin, Irinotecan (CAMPTOSAR® or CPT-11 or Camptothecin-11 or Campto), Carboplatin, edatrexate,

gemcitabine, or vinorelbine; radiation therapy, or analgesic therapy and/or any combination thereof.

In another embodiment, the invention features a composition comprising a nucleic acid molecule of the invention in a pharmaceutically acceptable carrier.

5 In one embodiment, the invention features a method of administering to a cell, for example a mammalian cell or human cell, a nucleic acid molecule of the invention comprising contacting the cell with the nucleic acid molecule under conditions suitable for administration. The method of administration can be in the presence of a delivery reagent, for example a lipid, cationic lipid, phospholipid, or liposome.

10

Detailed Description of the Invention

First the drawings will be described briefly.

Drawings

15 **Figure 1** shows examples of chemically stabilized ribozyme motifs. **HH Rz**, represents hammerhead ribozyme motif (Usman *et al.*, 1996, *Curr. Op. Struct. Bio.*, 1, 527); **NCH Rz** represents the NCH ribozyme motif (Ludwig *et al.*, International PCT Publication No. WO 98/58058 and US Patent Application Serial No. 08/878,640); **G-Cleaver**, represents G-cleaver ribozyme motif (Kore *et al.*, 1998, *Nucleic Acids Research* 26, 4116-4120, Eckstein *et al.*, US 6,127,173). **N** or **n**, represent independently a nucleotide which can be same or
20 different and have complementarity to each other; **rI**, represents ribo-Inosine nucleotide; arrow indicates the site of cleavage within the target. Position 4 of the HH Rz and the NCH Rz is shown as having 2'-C-allyl modification, but those skilled in the art will recognize that this position can be modified with other modifications well known in the art, so long as such modifications do not significantly inhibit the activity of the ribozyme.

25 **Figure 2** shows an example of the Amberzyme ribozyme motif that is chemically stabilized (see for example Beigelman *et al.*, International PCT publication No. WO 99/55857 and US Patent Application Serial No. 09/476,387.).

30 **Figure 3** shows an example of a Zinzyne A ribozyme motif that is chemically stabilized (see for example Beigelman *et al.*, International PCT publication No. WO 99/55857 and US Patent Application Serial No. 09/918,728).

Figure 4 shows an example of a DNAzyme motif described by Santoro *et al.*, 1997, *PNAS*, 94, 4262 and Joyce *et al.*, US 5,807,718.

5 The invention features novel nucleic acid molecules, including antisense oligonucleotides, siRNA and enzymatic nucleic acid molecules, and methods to modulate gene expression, for example, genes encoding K-Ras, H-Ras and/or N-Ras. In particular, the instant invention features nucleic-acid based molecules and methods to down-regulate the expression of K-Ras, H-Ras and/or N-Ras gene sequences.

10 The invention features one or more nucleic acid-based molecules and methods that independently or in combination modulate the expression of a gene or genes encoding Ras proteins. In particular embodiments, the invention features nucleic acid-based molecules and methods that modulate the expression of K-Ras gene, for example, Genbank Accession No. NM_004985; H-Ras gene, for example, Genbank Accession No. NM_005343; and/or N-Ras gene, for example, Genbank Accession No. NM_002524.

15 The description below of the various aspects and embodiments is provided with reference to exemplary K-Ras, H-Ras, and N-Ras genes, referred to hereinafter collectively as Ras. However, the various aspects and embodiments are directed to equivalent sequences and also to other genes which encode K-Ras, H-Ras and/or N-Ras proteins and similar proteins to K-Ras, H-Ras and/or N-Ras. For example, the invention relates to genes with homology to genes that encode K-Ras, H-Ras and/or N-Ras and genes that encode proteins with similar function to K-Ras, H-Ras, and N-Ras proteins. Those additional genes can be analyzed for target sites using the methods described herein. Thus, the modulation and the effects of such modulation of the other genes can be determined as described herein.

20 In one embodiment, the invention features the use of an enzymatic nucleic acid molecule, preferably in the hammerhead, NCH, G-cleaver, amberzyme, zinzyme and/or DNAzyme motif, to modulate the expression of a Ras gene or inhibit Ras activity. In one embodiment, the invention features the use of these enzymatic nucleic acid molecules to down-regulate the expression of a Ras gene or inhibit Ras activity. In another embodiment, the invention features the use of an antisense oligonucleotide molecule to modulate, for example, down-regulate, the expression of a Ras gene or inhibit Ras activity.

30 The invention features novel enzymatic nucleic acid molecules, siRNA molecules, and methods to modulate expression and/or activity of human immunodeficiency virus (HIV), for example HIV-1, HIV-2, and related viruses such as FIV-1 and SIV-1, or a HIV gene, for

example *LTR*, *nef*, *vif*, *tat*, or *rev*. In particular, the instant invention features nucleic-acid based molecules and methods to inhibit the replication of a HIV or related virus.

5 The invention features one or more nucleic acid-based molecules and methods that independently or in combination modulate the expression of gene(s) encoded by HIV and/or inhibit the replication of HIV. In particular embodiments, the invention features nucleic acid-based molecules and methods that modulate the expression of HIV-1 encoded genes, for example (Genbank Accession No. AJ302647); HIV-2 gene, for example (Genbank Accession No. NC_001722), FIV-1, for example (Genbank Accession No. NC_001482), SIV-1, for example (Genbank Accession No. M66437), *LTR*, for example included in (Genbank
10 Accession No. AJ302647), *nef*, for example included in (Genbank Accession No. AJ302647), *vif*, for example included in (Genbank Accession No. AJ302647), *tat*, for example included in (Genbank Accession No. AJ302647), and *rev*, for example included in (Genbank Accession No. AJ302647).

15 The description below of the various aspects and embodiments is provided with reference to the exemplary HIV-1 gene, referred to herein as HIV. However, the various aspects and embodiments are also directed to other genes which encode HIV proteins and similar viruses to HIV. Those additional genes can be analyzed for target sites using the methods described for HIV. Thus, the inhibition and the effects of such inhibition of the other genes can be performed as described herein.

20 Due to the high sequence variability of the HIV genome, selection of nucleic acid molecules for broad therapeutic applications would likely involve the conserved regions of the HIV genome. Specifically, the present invention describes nucleic acid molecules that cleave the conserved regions of the HIV genome. Therefore, one nucleic acid molecule can be designed to cleave all the different isolates of HIV. Nucleic acid molecules designed
25 against conserved regions of various HIV isolates can enable efficient inhibition of HIV replication in diverse subject populations and can ensure the effectiveness of the nucleic acid molecules against HIV quasi species which evolve due to mutations in the non-conserved regions of the HIV genome.

30 In one embodiment, the invention features the use of an enzymatic nucleic acid molecule, preferably in the hammerhead, NCH, G-cleaver, amberzyme, zinzyme and/or DNAzyme motif, to down-regulate the expression of HIV genes or inhibit the replication of HIV.

The invention features novel nucleic acid molecules, siRNA molecules and methods to modulate gene expression, for example, genes encoding HER2. In particular, the instant invention features nucleic-acid based molecules and methods to inhibit the expression of HER2.

5 The invention features one or more nucleic acid-based molecules and methods that independently or in combination modulate the expression of a gene or genes encoding HER2. In particular embodiments, the invention features nucleic acid-based molecules and methods that modulate the expression of HER2 gene, for example, Genbank Accession No. NM_004448.

10 The description below of the various aspects and embodiments is provided with reference to an exemplary HER2 gene, referred to herein as HER2 but also known as ERB2, ERB-B2, NEU, NGL, and v-ERB-B2. However, the various aspects and embodiments are also directed to other genes which encode HER2 proteins and similar proteins to HER2. Those additional genes can be analyzed for target sites using the methods described for
15 HER2. Thus, the inhibition and the effects of such inhibition of the other genes can be performed as described herein.

In one embodiment, the invention features the use of an enzymatic nucleic acid molecule, preferably in the hammerhead, NCH, G-cleaver, amberzyme, zinzyme and/or DNAzyme motif, to down-regulate the expression of HER2 genes or inhibit HER2 activity.

20 By "modulate" is meant that the expression of the gene, or level of RNAs or equivalent RNAs encoding one or more protein subunits or components, or activity of one or more proteins is up-regulated or down-regulated, such that the expression, level, or activity is greater than or less than that observed in the absence of the nucleic acid molecules of the invention.

25 By "inhibit" or "down-regulate" it is meant that the expression of the gene, or level of RNAs or equivalent RNAs encoding one or more protein subunits or components, or activity of one or more protein subunits or components, such as Ras, HIV, and/or HER2 protein or proteins, is reduced below that observed in the absence of the nucleic acid molecules of the invention. In one embodiment, inhibition or down-regulation with the enzymatic nucleic acid
30 molecule preferably is below that level observed in the presence of an enzymatically inactive or attenuated enzymatic nucleic acid molecule that is able to bind to the same site on the target RNA, but is unable to cleave that RNA. In another embodiment, inhibition or down-

regulation with an antisense oligonucleotide is preferably below that level observed in the presence of, for example, an oligonucleotide with scrambled sequence or with mismatches. In another embodiment, inhibition or down-regulation with an siRNA molecule is preferably below that level observed in the presence of, for example, an oligonucleotide with scrambled sequence or with mismatches. In another embodiment, inhibition or down-regulation of Ras, HIV, or HER2 expression and/or activity with the nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence.

By "up-regulate" is meant that the expression of the gene, or level of RNAs or equivalent RNAs encoding one or more protein subunits or components, or activity of one or more protein subunits or components, such as Ras, HIV, or HER2 protein or proteins, is greater than that observed in the absence of the nucleic acid molecules of the invention. For example, the expression of a gene, such as Ras, HIV, or HER2 gene, can be increased in order to treat, prevent, ameliorate, or modulate a pathological condition caused or exacerbated by an absence or low level of gene expression.

By "enzymatic nucleic acid molecule" as used herein, is meant a nucleic acid molecule which has complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity which is active to specifically cleave target RNA. That is, the enzymatic nucleic acid molecule is able to intermolecularly cleave RNA and thereby inactivate a target RNA molecule. These complementary regions allow sufficient hybridization of the enzymatic nucleic acid molecule to the target RNA and thus permit cleavage. One hundred percent complementarity is preferred, but complementarity as low as 50-75% can also be useful in this invention (see for example Werner and Uhlenbeck, 1995, *Nucleic Acids Research*, 23, 2092-2096; Hammann *et al.*, 1999, *Antisense and Nucleic Acid Drug Dev.*, 9, 25-31). The nucleic acids can be modified at the base, sugar, and/or phosphate groups. The term DNAzyme-based enzymatic nucleic acid is used interchangeably with phrases such as catalytic DNA, aptazyme or aptamer-binding DNAzyme, regulatable DNAzyme, catalytic oligonucleotides, nucleozyme, DNAzyme, endoribonuclease, endonuclease, minizyme, leadzyme, oligozyme or DNA enzyme. All of these terminologies describe nucleic acid molecules with enzymatic activity. The specific enzymatic nucleic acid molecules described in the instant application are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it have a specific substrate binding site which is complementary to one or more of the target nucleic acid regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart a nucleic acid cleaving and/or ligation activity to the molecule.

By "nucleic acid molecule" as used herein is meant a molecule having nucleotides. The nucleic acid can be single, double, or multiple stranded and can comprise modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof.

By "enzymatic portion" or "catalytic domain" is meant that portion/region of the enzymatic nucleic acid molecule essential for cleavage of a nucleic acid substrate (for example see **Figures 1-4**).

By "substrate binding arm" or "substrate binding domain" is meant that portion/region of a enzymatic nucleic acid which is able to interact, for example via complementarity (*i.e.*, able to base-pair with), with a portion of its substrate. Preferably, such complementarity is 100%, but can be less if desired. For example, as few as 10 bases out of 14 can be base-paired (see for example Werner and Uhlenbeck, 1995, *Nucleic Acids Research*, 23, 2092-2096; Hammann *et al.*, 1999, *Antisense and Nucleic Acid Drug Dev.*, 9, 25-31). Examples of such arms are shown generally in **Figures 1-3**. That is, these arms contain sequences within a enzymatic nucleic acid which are intended to bring enzymatic nucleic acid and target RNA together through complementary base-pairing interactions. The enzymatic nucleic acid of the invention can have binding arms that are contiguous or non-contiguous and can be of varying lengths. The length of the binding arm(s) are preferably greater than or equal to four nucleotides and of sufficient length to stably interact with the target RNA; preferably 12-100 nucleotides; more preferably 14-24 nucleotides long (see for example Werner and Uhlenbeck, *supra*; Hamman *et al.*, *supra*; Hampel *et al.*, EP0360257; Berzal-Herranz *et al.*, 1993, *EMBO J.*, 12, 2567-73). If two binding arms are chosen, the design is such that the length of the binding arms are symmetrical (*i.e.*, each of the binding arms is of the same length; *e.g.*, five and five nucleotides, or six and six nucleotides, or seven and seven nucleotides long) or asymmetrical (*i.e.*, the binding arms are of different length; *e.g.*, six and three nucleotides; three and six nucleotides long; four and five nucleotides long; four and six nucleotides long; four and seven nucleotides long; and the like).

By "Inozyme" or "NCH" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described as NCH Rz in **Figure 1** and in Ludwig *et al.*, International PCT Publication No. WO 98/58058 and US Patent Application Serial No. 08/878,640. Inozymes possess endonuclease activity to cleave nucleic acid substrates having a cleavage triplet NCH/, where N is a nucleotide, C is cytidine and H is adenosine, uridine or cytidine, and "/" represents the cleavage site. H is used interchangeably with X. Inozymes can also possess endonuclease activity to cleave nucleic acid substrates having a cleavage triplet NCN/, where N is a nucleotide, C is cytidine, and "/" represents the cleavage site. "T"

in Figure 1 represents an Inosine nucleotide, preferably a ribo-Inosine or xylo-Inosine nucleoside.

By "G-cleaver" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described as G-cleaver Rz in Figure 1 and in Eckstein *et al.*, US 6,127,173. G-cleavers possess endonuclease activity to cleave nucleic acid substrates having a cleavage triplet NYN/, where N is a nucleotide, Y is uridine or cytidine and "/" represents the cleavage site. G-cleavers can be chemically modified as is generally shown in Figure 1.

By "amberzyme" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described in Figure 2 and in Beigelman *et al.*, International PCT publication No. WO 99/55857 and US Patent Application Serial No. 09/476,387. Amberzymes possess endonuclease activity to cleave nucleic acid substrates having a cleavage triplet NG/N, where N is a nucleotide, G is guanosine, and "/" represents the cleavage site. Amberzymes can be chemically modified to increase nuclease stability through substitutions as are generally shown in Figure 2. In addition, differing nucleoside and/or non-nucleoside linkers can be used to substitute the 5'-gaaa-3' loops shown in the figure. Amberzymes represent a non-limiting example of an enzymatic nucleic acid molecule that does not require a ribonucleotide (2'-OH) group within its own nucleic acid sequence for activity.

By "zinzyme" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described in Figure 3 and in Beigelman *et al.*, International PCT publication No. WO 99/55857 and US Patent Application Serial No. 09/918,728. Zinzymes possess endonuclease activity to cleave nucleic acid substrates having a cleavage triplet including but not limited to YG/Y, where Y is uridine or cytidine, and G is guanosine and "/" represents the cleavage site. Zinzymes can be chemically modified to increase nuclease stability through substitutions as are generally shown in Figure 3, including substituting 2'-O-methyl guanosine nucleotides for guanosine nucleotides. In addition, differing nucleotide and/or non-nucleotide linkers can be used to substitute the 5'-gaaa-2' loop shown in the figure. Zinzymes represent a non-limiting example of an enzymatic nucleic acid molecule that does not require a ribonucleotide (2'-OH) group within its own nucleic acid sequence for activity.

By 'DNAzyme' is meant, an enzymatic nucleic acid molecule that does not require the presence of a 2'-OH group within its own nucleic acid sequence for activity. In particular

embodiments the enzymatic nucleic acid molecule can have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. DNAzymes can be synthesized chemically or expressed endogenously *in vivo*, by means of a single stranded DNA vector or equivalent thereof. An example of a DNAzyme is shown in Figure 4 and is generally reviewed in Usman *et al.*, US patent No., 6,159,714; Chartrand *et al.*, 1995, *NAR* 23, 4092; Breaker *et al.*, 1995, *Chem. Bio.* 2, 655; Santoro *et al.*, 1997, *PNAS* 94, 4262; Breaker, 1999, *Nature Biotechnology*, 17, 422-423; and Santoro *et al.*, 2000, *J. Am. Chem. Soc.*, 122, 2433-39. The "10-23" DNAzyme motif is one particular type of DNAzyme that was evolved using *in vitro* selection, see Santoro *et al.*, *supra* and as generally described in Joyce *et al.*, US 5,807,718. Additional DNAzyme motifs can be selected by using techniques similar to those described in these references, and hence, are within the scope of the present invention. DNAzymes of the invention can comprise nucleotides modified at the nucleic acid base, sugar, or phosphate backbone. Non-limiting examples of sugar modifications that can be used in DNAzymes of the invention include 2'-O-alkyl modifications such as 2'-O-methyl or 2'-O-allyl, 2'-C-alkyl modifications such as 2'-C-allyl, 2'-deoxy-2'-amino, 2'-halo modifications such as 2'-fluoro, 2'-chloro, or 2'-bromo, isomeric modifications such as arabinofuranose or xylofuranose based nucleic acids, and other sugar modifications such as 4'-thio or 4'-carbocyclic nucleic acids. Non-limiting examples of nucleic acid based modifications that can be used in DNAzymes of the invention include modified purine heterocycles, G-clamp heterocycles, and various modified pyrimidine cycles. Non-limiting examples of backbone modifications that can be used in DNAzymes of the invention include phosphorothioate, phosphorodithioate, phosphoramidate, and methylphosphonate internucleotide linkages. DNAzymes of the invention can comprise naturally occurring nucleic acids, chimeras of chemically modified and naturally occurring nucleic acids, or completely modified nucleic acids.

In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid that is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets. Thus, a single enzymatic nucleic acid molecule is able to cleave many molecules of target RNA. In addition, the enzymatic nucleic acid molecule is a highly

specific inhibitor of gene expression, with the specificity of inhibition depending not only on the base-pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of an enzymatic nucleic acid molecule.

5 By "sufficient length" is meant an oligonucleotide of greater than or equal to 3 nucleotides that is of a length great enough to provide the intended function under the expected condition. For example, for binding arms of enzymatic nucleic acid "sufficient length" means that the binding arm sequence is long enough to provide stable binding to a target site under the expected binding conditions. Preferably, the binding arms are not so
10 long as to prevent useful turnover of the nucleic acid molecule.

By "stably interact" is meant interaction of oligonucleotides with target nucleic acid molecules (*e.g.*, by forming hydrogen bonds with complementary nucleotides in the target under physiological conditions) that is sufficient to the intended purpose (*e.g.*, cleavage of target RNA by an enzyme).

15 By "equivalent" RNA to Ras is meant to include those naturally occurring RNA molecules having homology (partial or complete) to Ras nucleic acids or encoding for proteins with similar function as Ras proteins in various organisms, including humans, rodents, primates, rabbits, pigs, protozoans, fungi, plants, and other microorganisms and parasites. The equivalent RNA sequence can also include, in addition to the coding region,
20 regions such as a 5'-untranslated region, a 3'-untranslated region, introns, a intron-exon junction and the like.

By "equivalent" RNA to HIV is meant to include those naturally occurring RNA molecules having homology (partial or complete) to HIV nucleic acids or encoding for proteins with similar function as HIV proteins in various organisms, including human, rodent,
25 primate, rabbit, pig, protozoans, fungi, plants, and other microorganisms and parasites. The equivalent RNA sequence also includes in addition to the coding region, regions such as 5'-untranslated region, 3'-untranslated region, introns, intron-exon junction and the like.

By "equivalent" RNA to HER2 is meant to include those naturally occurring RNA molecules having homology (partial or complete) to HER2 nucleic acids or encoding for proteins with similar function as HER2 proteins in various organisms, including humans,
30 rodents, primates, rabbits, pigs, protozoans, fungi, plants, and other microorganisms and parasites. The equivalent RNA sequence also includes, in addition to the coding region,

regions such as a 5'-untranslated region, a 3'-untranslated region, introns, a intron-exon junction and the like.

By "homology" is meant the nucleotide sequence of two or more nucleic acid molecules is partially or completely identical.

5 By "component" of HIV is meant a peptide or protein expressed from an HIV gene, for example *nef*, *vif*, *tat*, or *rev* viral gene products.

By "component" of HER2 is meant a peptide or protein subunit expressed from a HER2 gene.

10 By "component" of Ras is meant a peptide or protein subunit expressed from a Ras gene.

By "gene" it is meant a nucleic acid that encodes an RNA, for example, nucleic acid sequences including but not limited to structural genes encoding a polypeptide.

"Complementarity" refers to the ability of a nucleic acid to form hydrogen bond or bonds with another RNA sequence by either traditional Watson-Crick or other non-traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its target or complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., enzymatic nucleic acid cleavage, antisense or triple helix inhibition. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner *et al.*, 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier *et al.*, 1986, *Proc. Nat. Acad. Sci. USA* 83:9373-9377; Turner *et al.*, 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" or "2'-OH" is meant a nucleotide with a hydroxyl group at the 2' position of a β -D-ribo-furanose moiety.

30 By "decoy" is meant a nucleic acid molecule, for example RNA or DNA, or aptamer that is designed to preferentially bind to a predetermined ligand. Such binding can result in

the inhibition or activation of a target molecule. A decoy or aptamer can compete with a naturally occurring binding target for the binding of a specific ligand. For example, it has been shown that over-expression of HIV trans-activation response (TAR) RNA can act as a "decoy" and efficiently binds HIV tat protein, thereby preventing it from binding to TAR sequences encoded in the HIV RNA (Sullenger *et al.*, 1990, *Cell*, 63, 601-608). This is but a specific example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art, see for example Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science*, 287, 820; and Jayasena, 1999, *Clinical Chemistry*, 45, 1628. Similarly, a decoy can be designed to bind to Ras and block the binding of Ras or a decoy can be designed to bind to Ras and prevent interaction with the Ras protein.

By "aptamer" or "nucleic acid aptamer" as used herein is meant a nucleic acid molecule that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that is distinct from sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. Similarly, the nucleic acid molecules of the instant invention can bind to RAS, Her-2 or HIV encoded RNA or proteins receptors to block activity of the activity of target protein or nucleic acid. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art, see for example Gold *et al.*, US 5,475,096 and 5,270,163; Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science*, 287, 820; and Jayasena, 1999, *Clinical Chemistry*, 45, 1628.

The term "short interfering RNA" or "siRNA" as used herein refers to a double stranded nucleic acid molecule capable of RNA interference "RNAi", see for example Bass, 2001, *Nature*, 411, 428-429; Elbashir *et al.*, 2001, *Nature*, 411, 494-498; and Kreutzer *et al.*, International PCT Publication No. WO 00/44895; Zernicka-Goetz *et al.*, International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck *et al.*, International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li *et al.*, International PCT Publication No. WO

00/44914. As used herein, siRNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically modified nucleotides and non-nucleotides.

5 Nucleic acid molecules that modulate expression of Ras-specific RNAs represent a therapeutic approach to treat cancer, including, but not limited to colorectal cancer, bladder cancer, lung cancer, pancreatic cancer, breast cancer, or prostate cancer and any other cancer, disease or condition that responds to the modulation of Ras expression.

10 Nucleic acid molecules that modulate expression of HIV-specific RNAs also represent a therapeutic approach to treat acquired immunodeficiency syndrome (AIDS) and/or any other disease, condition, or syndrome which respond to the modulation of HIV expression.

Nucleic acid molecules that modulate expression of HER2-specific RNAs represent a therapeutic approach to treat cancer, including, but not limited to breast and ovarian cancer and any other cancer, disease or condition that responds to the modulation of HER2 expression.

15 In one embodiment of the inventions described herein, the enzymatic nucleic acid molecule is formed in a hammerhead or hairpin motif, but can also be formed in the motif of a hepatitis delta virus, group I intron, group II intron or RNase P RNA (in association with an RNA guide sequence), *Neurospora* VS RNA, DNAzymes, NCH cleaving motifs, or G-cleavers. Examples of such hammerhead motifs are described by Dreyfus, *supra*, Rossi *et al.*,
20 1992, *AIDS Research and Human Retroviruses* 8, 183; of hairpin motifs by Hampel *et al.*, EP0360257, Hampel and Tritz, 1989 *Biochemistry* 28, 4929, Feldstein *et al.*, 1989, *Gene* 82, 53, Haseloff and Gerlach, 1989, *Gene*, 82, 43, and Hampel *et al.*, 1990 *Nucleic Acids Res.* 18, 299; Chowrira & McSwiggen, US. Patent No. 5,631,359; of the hepatitis delta virus motif is described by Perrotta and Been, 1992 *Biochemistry* 31, 16; of the RNase P motif by Guerrier-
25 Takada *et al.*, 1983 *Cell* 35, 849; Forster and Altman, 1990, *Science* 249, 783; Li and Altman, 1996, *Nucleic Acids Res.* 24, 835; *Neurospora* VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990 *Cell* 61, 685-696; Saville and Collins, 1991 *Proc. Natl. Acad. Sci. USA* 88, 8826-8830; Collins and Olive, 1993 *Biochemistry* 32, 2795-2799; Guo and Collins, 1995, *EMBO. J.* 14, 363); Group II introns are described by Griffin *et al.*, 1995,
30 *Chem. Biol.* 2, 761; Michels and Pyle, 1995, *Biochemistry* 34, 2965; Pyle *et al.*, International PCT Publication No. WO 96/22689; of the Group I intron by Cech *et al.*, U.S. Patent 4,987,071 and of DNAzymes by Usman *et al.*, International PCT Publication No. WO 95/11304; Chartrand *et al.*, 1995, *NAR* 23, 4092; Breaker *et al.*, 1995, *Chem. Bio.* 2, 655; Santoro *et al.*, 1997, *PNAS* 94, 4262, and Beigelman *et al.*, International PCT publication No.

WO 99/55857. NCH cleaving motifs are described in Ludwig & Sproat, International PCT Publication No. WO 98/58058; and G-cleavers are described in Kore *et al.*, 1998, *Nucleic Acids Research* 26, 4116-4120 and Eckstein *et al.*, International PCT Publication No. WO 99/16871. Additional motifs such as the Aptazyme (Breaker *et al.*, WO 98/43993),
5 Amberzyme (Class I motif; Figure 2; Beigelman *et al.*, U.S. Serial No. 09/301,511) and Zinzyme (Figure 3) (Beigelman *et al.*, U.S. Serial No. 09/301,511), all included by reference herein including drawings, can also be used in the present invention. These specific motifs or configurations are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it has a
10 specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule (Cech *et al.*, U.S. Patent No. 4,987,071).

In one embodiment of the present invention, a nucleic acid molecule of the instant
15 invention can be between about 10 and 100 nucleotides in length. Exemplary enzymatic nucleic acid molecules of the invention are shown in the Tables herein. For example, enzymatic nucleic acid molecules of the invention are preferably between about 15 and 50 nucleotides in length, more preferably between about 25 and 40 nucleotides in length, *e.g.*, 34, 36, or 38 nucleotides in length (for example see Jarvis *et al.*, 1996, *J. Biol. Chem.*, 271, 29107-29112). Exemplary DNAzymes of the invention are preferably between about 15 and
20 40 nucleotides in length, more preferably between about 25 and 35 nucleotides in length, *e.g.*, 29, 30, 31, or 32 nucleotides in length (see for example Santoro *et al.*, 1998, *Biochemistry*, 37, 13330-13342; Chartrand *et al.*, 1995, *Nucleic Acids Research*, 23, 4092-4096). Exemplary antisense molecules of the invention are preferably between about 15 and 75
25 nucleotides in length, more preferably between about 20 and 35 nucleotides in length, *e.g.*, 25, 26, 27, or 28 nucleotides in length (see for example Woolf *et al.*, 1992, *PNAS*, 89, 7305-7309; Milner *et al.*, 1997, *Nature Biotechnology*, 15, 537-541). Exemplary triplex forming oligonucleotide molecules of the invention are preferably between about 10 and 40 nucleotides in length, more preferably between about 12 and 25 nucleotides in length, *e.g.*,
30 18, 19, 20, or 21 nucleotides in length (see for example Maher *et al.*, 1990, *Biochemistry*, 29, 8820-8826; Strobel and Dervan, 1990, *Science*, 249, 73-75). Those skilled in the art will recognize that all that is required is for a nucleic acid molecule to be of length and conformation sufficient and suitable for the nucleic acid molecule to interact with its target and/or catalyze a reaction contemplated herein. The length of nucleic acid molecules of the
35 instant invention are not limiting within the general limits stated.

Preferably, a nucleic acid molecule that modulates, for example, down-regulates Ras, HIV, and/or HER2 expression and/or activity, comprises between 12 and 100 bases complementary to a RNA molecule of Ras, HIV, and/or HER2 respectively. Even more preferably, a nucleic acid molecule that modulates Ras, HIV, and/or HER2 expression
5 comprises between 14 and 24 bases complementary to a RNA molecule of Ras, HIV, and/or HER2 respectively.

The invention provides a method for producing a class of nucleic acid-based gene modulating agents that exhibit a high degree of specificity for RNA of a desired target. For example, an enzymatic nucleic acid molecule is preferably targeted to a highly conserved
10 sequence region of target RNAs encoding Ras (and specifically a Ras gene) such that specific treatment of a disease or condition can be provided with either one or several nucleic acid molecules of the invention. Such nucleic acid molecules can be delivered exogenously to specific tissue or cellular targets as required. Alternatively, the nucleic acid molecules (e.g., enzymatic nucleic acid molecules, siRNA, antisense, and/or DNazymes) can be expressed
15 from DNA and/or RNA vectors that are delivered to specific cells.

As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism. A cell can, for example, be *in vitro*, e.g., in cell culture, or present in a multicellular organism, including, e.g., birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (e.g., bacterial cell)
20 or eukaryotic (e.g., mammalian or plant cell).

By "Ras proteins" is meant, a peptide or protein comprising Ras tyrosine kinase-type cell surface receptor or a peptide or protein encoded by a Ras gene, such as K-Ras, H-Ras, or N-Ras.

By "HIV proteins" is meant, a peptide or protein comprising a component of HIV or a
25 peptide or protein encoded by a HIV gene.

By "HER2 proteins" is meant, a peptide or protein comprising HER2/ERB2/NEU tyrosine kinase-type cell surface receptor or a peptide or protein encoded by a HER2/ERB2/NEU gene.

By "highly conserved sequence region" is meant, a nucleotide sequence of one or more
30 regions in a target gene that does not vary significantly from one generation to the other or from one biological system to the other.

Nucleic acid-based modulators, including inhibitors, of Ras expression are useful for the prevention and/or treatment of cancer, including but not limited to breast cancer and ovarian cancer and any other disease or condition that respond to the modulation of Ras expression.

- 5 Nucleic acid-based inhibitors of HIV expression are useful for the prevention and/or treatment of acquired immunodeficiency disease (AIDS) and related diseases and conditions, including but not limited to Kaposi's sarcoma, lymphoma, cervical cancer, squamous cell carcinoma, cardiac myopathy, rheumatic diseases, and opportunistic infection, for example Pneumocystis carinii, Cytomegalovirus, Herpes simplex, Mycobacteria, Cryptococcus,
- 10 Toxoplasma, Progressive multifocal leucoencephalopathy (Papovavirus), Mycobacteria, Aspergillus, Cryptococcus, Candida, Cryptosporidium, Isospora belli, Microsporidia and any other disease or condition which respond to the modulation of HIV expression.

- Nucleic acid-based inhibitors of HER2 expression are useful for the prevention and/or treatment of cancer, including but not limited to breast cancer and ovarian cancer and any
- 15 other disease or condition that respond to the modulation of HER2 expression.

By "related" is meant that the reduction of RAS, HIV, or HER2 expression (specifically RAS, HIV, or HER2 genes respectively) RNA levels and thus reduction in the level of the respective protein relieves, to some extent, the symptoms of the disease or condition.

- The nucleic acid-based molecules of the invention can be added directly, or can be
- 20 complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection or infusion pump, with or without their incorporation in biopolymers. In certain embodiments, the enzymatic nucleic acid molecules comprise sequences that are complementary to the substrate sequences in the Tables herein.
- 25 Examples of such enzymatic nucleic acid molecules also are shown in the Tables herein. Examples of such enzymatic nucleic acid molecules consist essentially of sequences defined in these tables.

- In another embodiment, the invention features siRNA, antisense nucleic acid molecules and 2-5A chimeras comprising sequences complementary to the substrate sequences shown in
- 30 the Tables herein. Such nucleic acid molecules can comprise sequences as shown for the binding arms of the enzymatic nucleic acid molecules in the Tables. Similarly, triplex molecules can be targeted to corresponding DNA target regions; such molecules can comprise the DNA equivalent of a target sequence or a sequence complementary to the specified target

(substrate) sequence. Typically, antisense molecules are complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule can bind to a substrate such that the substrate molecule forms a loop, and/or an antisense molecule can bind such that the antisense molecule forms a loop. Thus, the antisense molecule can be complementary to two or more non-contiguous substrate sequences. In addition, two or more non-contiguous sequence portions of an antisense molecule can be complementary to a target sequence.

By "consists essentially of" is meant that the active nucleic acid molecule of the invention, for example, an enzymatic nucleic acid molecule, contains an enzymatic center or core equivalent to those in the examples, and binding arms able to bind RNA such that cleavage at the target site occurs. Other sequences can be present that do not interfere with such cleavage. Thus, a core region of an enzymatic nucleic acid molecule can, for example, include one or more loop, stem-loop structure, or linker that does not prevent enzymatic activity. Thus, various regions in the sequences in the Tables can be such a loop, stem-loop, nucleotide linker, and/or non-nucleotide linker and can be represented generally as sequence "X". The nucleic acid molecules of the instant invention, such as Hammerhead, Inozyme, G-cleaver, amberzyme, zinzyme, DNAzyme, antisense, 2-5A antisense, triplex forming nucleic acid, and decoy nucleic acids, can contain other sequences or non-nucleotide linkers that do not interfere with the function of the nucleic acid molecule.

Sequence X can be a linker of ≥ 2 nucleotides in length, preferably 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 26, 30, where the nucleotides can preferably be internally base-paired to form a stem of preferably ≥ 2 base pairs. Alternatively or in addition, sequence X can be a non-nucleotide linker. In yet another embodiment, the nucleotide linker X can be a nucleic acid aptamer, such as an ATP aptamer, Ras Rev aptamer (RRE), Ras Tat aptamer (TAR) and others (for a review see Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; and Szostak & Ellington, 1993, in *The RNA World*, ed. Gesteland and Atkins, pp. 511, CSH Laboratory Press). A "nucleic acid aptamer" as used herein is meant to indicate a nucleic acid sequence capable of interacting with a ligand. The ligand can be any natural or a synthetic molecule, including but not limited to a resin, metabolites, nucleosides, nucleotides, drugs, toxins, transition state analogs, peptides, lipids, proteins, amino acids, nucleic acid molecules, hormones, carbohydrates, receptors, cells, viruses, bacteria and others.

In yet another embodiment, a non-nucleotide linker X is as defined herein. Non-nucleotides as can include abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, or polyhydrocarbon compounds. Specific examples include those

described by Seela and Kaiser, *Nucleic Acids Res.* 1990, 18:6353 and *Nucleic Acids Res.* 1987, 15:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, 113:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, 113:5109; Ma *et al.*, *Nucleic Acids Res.* 1993, 21:2585 and *Biochemistry* 1993, 32:1751; Durand *et al.*, *Nucleic Acids Res.* 1990, 18:6353; McCurdy
5 *et al.*, *Nucleosides & Nucleotides* 1991, 10:287; Jsche *et al.*, *Tetrahedron Lett.* 1993, 34:301; Ono *et al.*, *Biochemistry* 1991, 30:9914; Arnold *et al.*, International Publication No. WO 89/02439; Usman *et al.*, International Publication No. WO 95/06731; Dudycz *et al.*, International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, 113:4000, all hereby incorporated by reference herein. A "non-nucleotide" further
10 means any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine. Thus, in a preferred embodiment, the
15 invention features an enzymatic nucleic acid molecule having one or more non-nucleotide moieties, and having enzymatic activity to cleave an RNA or DNA molecule.

In another aspect of the invention, enzymatic nucleic acid molecules, siRNA molecules or antisense molecules that interact with target RNA molecules and modulate gene expression activity are expressed from transcription units inserted into DNA or RNA vectors. The
20 recombinant vectors are preferably DNA plasmids or viral vectors. Enzymatic nucleic acid molecule or antisense expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus as well as others known in the art. Preferably, recombinant vectors capable of expressing enzymatic nucleic acid molecules or antisense are delivered as described below, and persist in target cells. Alternatively, viral
25 vectors can be used that provide for transient expression of enzymatic nucleic acid molecules or antisense. Such vectors can be repeatedly administered as necessary. Once expressed, the enzymatic nucleic acid molecules or antisense bind to target RNA and modulate its function or expression. Delivery of enzymatic nucleic acid molecule or antisense expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to
30 target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that allows for introduction into a desired target cell. Antisense DNA and DNAzymes can be expressed via the use of a single stranded DNA intracellular expression vector.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a
35 desired nucleic acid.

By "subject" or "patient" is meant an organism that is a donor or recipient of explanted cells or the cells of the organism. "Subject" or "patient" also refers to an organism to which the nucleic acid molecules of the invention can be administered. Preferably, a subject or patient is a mammal or mammalian cells. More preferably, a subject or patient is a human or human cells.

By "enhanced enzymatic activity" is meant to include activity measured in cells and/or *in vivo* where the activity is a reflection of both the catalytic activity and the stability of the nucleic acid molecules of the invention. In this invention, the product of these properties can be increased *in vivo* compared to an all RNA enzymatic nucleic acid or all DNA enzyme, for example, with a nucleic acid molecule comprising chemical modifications. In some cases, the activity or stability of the nucleic acid molecule can be decreased (i.e., less than ten-fold), but the overall activity of the nucleic acid molecule is enhanced, *in vivo*.

Nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed above. For example, to treat a disease or condition associated with the levels of Ras, HIV, or HER2, a subject can be treated, or other appropriate cells can be treated, as is evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

In a further embodiment, the described molecules, such as antisense, siRNA, or enzymatic nucleic acid molecules, can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described molecules can be used in combination with one or more known therapeutic agents to treat cancer, for example colorectal cancer, bladder cancer, lung cancer, pancreatic cancer, breast cancer, or prostate cancer, and any other disease or condition that respond to the modulation of Ras expression.

In another embodiment, the invention features nucleic acid-based inhibitors (e.g., enzymatic nucleic acid molecules, (including DNazymes), siRNA and methods for their use to down regulate or inhibit the expression of genes (e.g., Ras genes) capable of progression and/or maintenance of cancer and/or other disease states that respond to the modulation of Ras expression.

In a further embodiment, the described molecules, such as antisense, siRNA, or enzymatic nucleic acids, can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described molecules can be used in combination with one or more known therapeutic agents to treat acquired immunodeficiency

disease (AIDS) and related diseases and conditions, including but not limited to Kaposi's sarcoma, lymphoma, cervical cancer, squamous cell carcinoma, cardiac myopathy, rheumatic diseases, and opportunistic infection, for example *Pneumocystis carinii*, Cytomegalovirus, Herpes simplex, Mycobacteria, Cryptococcus, Toxoplasma, Progressive multifocal
5 leucoencephalopathy (Papovavirus), Mycobacteria, Aspergillus, Cryptococcus, Candida, Cryptosporidium, *Isospora belli*, Microsporidia and any other disease or condition which respond to the modulation of HIV expression.

Nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed above. For
10 example, to treat a disease or condition associated with the levels of HER2, a patient can be treated, or other appropriate cells can be treated, as is evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

In a further embodiment, the described molecules, such as antisense, siRNA or enzymatic nucleic acid molecules, can be used in combination with other known treatments to
15 treat conditions or diseases discussed above. For example, the described molecules can be used in combination with one or more known therapeutic agents to treat cancer, for example ovarian cancer and/or breast cancer, and any other disease or condition that respond to the modulation of HER2 expression.

In another embodiment, the invention features nucleic acid-based inhibitors (e.g., enzymatic nucleic acid molecules, (including ribozymes, antisense nucleic acids, 2-5A antisense chimeras, triplex DNA, antisense nucleic acids containing RNA cleaving chemical groups), siRNA and methods for their use to down regulate or inhibit the expression of genes
20 (e.g., HER2 genes) capable of progression and/or maintenance of cancer and/or other disease states that respond to the modulation of HER2 expression.
25

By "comprising" is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting
30 of".

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Mechanism of action of Nucleic Acid Molecules of the Invention as is Known in the Art

Antisense: Antisense molecules can be modified or unmodified RNA, DNA, or mixed polymer oligonucleotides and primarily function by specifically binding to matching sequences resulting in inhibition of peptide synthesis (Wu-Pong, Nov 1994, *BioPharm*, 20-33). The antisense oligonucleotide binds to target RNA by Watson Crick base-pairing and blocks gene expression by preventing ribosomal translation of the bound sequences either by steric blocking or by activating RNase H enzyme. Antisense molecules can also alter protein synthesis by interfering with RNA processing or transport from the nucleus into the cytoplasm (Mukhopadhyay & Roth, 1996, *Crit. Rev. in Oncogenesis* 7, 151-190).

10 In addition, binding of single stranded DNA to RNA can result in nuclease degradation of the heteroduplex (Wu-Pong, *supra*; Crooke, *supra*). Backbone modified DNA chemistry which have been thus far been shown to act as substrates for RNase H are phosphorothioates, phosphorodithioates, and borontrifluoridates. In addition, 2'-arabino and 2'-fluoro arabino-containing oligos can also activate RNase H activity.

15 A number of antisense molecules have been described that utilize novel configurations of chemically modified nucleotides, secondary structure, and/or RNase H substrate domains (Woelf *et al.*, International PCT Publication No. WO 98/13526; Thompson *et al.*, International PCT Publication No. WO 99/54459; Hartmann *et al.*, USSN 60/101,174, filed on September 21, 1998). All of these references are incorporated by reference herein in their
20 entirety.

In addition, antisense deoxyoligoribonucleotides can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which digests the target RNA in the duplex. Antisense DNA can be expressed via the use of a single stranded DNA intracellular expression vector or equivalents and variations thereof.

25 **RNA interference:** RNA interference refers to the process of sequence specific post transcriptional gene silencing in animals mediated by short interfering RNAs (siRNA) (Fire *et al.*, 1998, *Nature*, 391, 806). The corresponding process in plants is commonly referred to as post transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post transcriptional gene silencing is thought to be an evolutionarily
30 conserved cellular defense mechanism used to prevent the expression of foreign genes which is commonly shared by diverse flora and phyla (Fire *et al.*, 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double stranded RNAs (dsRNA) derived from viral infection or the random integration of

transposon elements into a host genome via a cellular response that specifically destroys homologous single stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response through a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA mediated activation of protein kinase PKR and 2',5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNA) (Berstein *et al.*, 2001, *Nature*, 409, 363). Short interfering RNAs derived from dicer activity are typically about 21-23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21 and 22 nucleotide small temporal RNAs (stRNA) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.*, 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the guide sequence of the siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188).

Short interfering RNA mediated RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature*, 391, 806, were the first to observe RNAi in *C. Elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describes RNAi mediated by dsRNA in mouse embryos. Hammond *et al.*, 2000, *Nature*, 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.*, 2001, *Nature*, 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in *Drosophila* embryonic lysates has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21 nucleotide siRNA duplexes are most active when containing two nucleotide 3'-overhangs. Furthermore, substitution of one or both siRNA strands with 2'-deoxy or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of 3'-terminal siRNA nucleotides with deoxy nucleotides was shown to be tolerated. Mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain

the 5'-phosphate moiety on the siRNA (Nykanen *et al.*, 2001, *Cell*, 107, 309), however siRNA molecules lacking a 5'-phosphate are active when introduced exogenously, suggesting that 5'-phosphorylation of siRNA constructs may occur *in vivo*.

Enzymatic Nucleic Acid: Several varieties of naturally-occurring enzymatic RNAs are presently known. In addition, several *in vitro* selection (evolution) strategies (Orgel, 1979, *Proc. R. Soc. London*, B 205, 435) have been used to evolve new nucleic acid catalysts capable of catalyzing cleavage and ligation of phosphodiester linkages (Joyce, 1989, *Gene*, 82, 83-87; Beaudry *et al.*, 1992, *Science* 257, 635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker *et al.*, 1994, *TIBTECH* 12, 268; Bartel *et al.*, 1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar *et al.*, 1995, *FASEB J.*, 9, 1183; Breaker, 1996, *Curr. Op. Biotech.*, 7, 442; Santoro *et al.*, 1997, *Proc. Natl. Acad. Sci.*, 94, 4262; Tang *et al.*, 1997, *RNA* 3, 914; Nakamaye & Eckstein, 1994, *supra*; Long & Uhlenbeck, 1994, *supra*; Ishizaka *et al.*, 1995, *supra*; Vaish *et al.*, 1997, *Biochemistry* 36, 6495; all of these are incorporated by reference herein). Each can catalyze a series of reactions including the hydrolysis of phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions.

Nucleic acid molecules of this invention can modulate, e.g., down-regulate, Ras protein expression and can be used to treat disease or diagnose disease associated with the levels of Ras, HIV and/or HER2. Enzymatic nucleic acid sequences targeting Ras, HIV and/or HER2 RNA and sequences that can be targeted with nucleic acid molecules of the invention to down-regulate Ras expression are shown in the Tables herein.

The enzymatic nature of an enzymatic nucleic acid molecule allows the concentration of enzymatic nucleic acid molecule necessary to affect a therapeutic treatment to be lower than a nucleic acid molecule lacking enzymatic activity. This reflects the ability of the enzymatic nucleic acid molecule to act enzymatically. Thus, a single enzymatic nucleic acid molecule is able to cleave many molecules of target RNA. In addition, the enzymatic nucleic acid molecule is a highly specific inhibitor, with the specificity of inhibition depending not only on the base-pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can be chosen to completely eliminate catalytic activity of a enzymatic nucleic acid molecule.

Nucleic acid molecules having an endonuclease enzymatic activity are able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence-specific manner. With proper design and construction, such enzymatic nucleic acid molecules can be targeted to virtually any RNA transcript, and achieve efficient cleavage *in vitro* (Zaug *et al.*,

324, *Nature* 429 1986; Uhlenbeck, 1987 *Nature* 328, 596; Kim *et al.*, 84 *Proc. Natl. Acad. Sci. USA* 8788, 1987; Dreyfus, 1988, *Einstein Quart. J. Bio. Med.*, 6, 92; Haseloff and Gerlach, 334 *Nature* 585, 1988; Cech, 260 *JAMA* 3030, 1988; and Jefferies *et al.*, 17 *Nucleic Acids Research* 1371, 1989; Santoro *et al.*, 1997 *supra*).

5 Because of their sequence specificity, *trans*-cleaving enzymatic nucleic acid molecules can be used as therapeutic agents for human disease (Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* 30, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* 38, 2023-2037). Enzymatic nucleic acid molecules can be designed to cleave specific RNA targets within the background of cellular RNA. Such a cleavage event renders the RNA non-functional and
10 abrogates protein expression from that RNA. In this manner, synthesis of a protein associated with a disease state can be selectively inhibited (Warashina *et al.*, 1999, *Chemistry and Biology*, 6, 237-250).

Enzymatic nucleic acid molecules of the invention that are allosterically regulated ("allozymes") can be used to modulate, including down-regulate, Ras, HIV and/or HER2
15 expression. These allosteric enzymatic nucleic acids or allozymes (see for example George *et al.*, US Patent Nos. 5,834,186 and 5,741,679, Shih *et al.*, US Patent No. 5,589,332, Nathan *et al.*, US Patent No 5,871,914, Nathan and Ellington, International PCT publication No. WO 00/24931, Breaker *et al.*, International PCT Publication Nos. WO 00/26226 and 98/27104, and Sullenger *et al.*, International PCT publication No. WO 99/29842) are designed to
20 respond to a signaling agent, for example, mutant Ras, HIV and/or HER2 protein, wild-type Ras, HIV and/or HER2 protein, mutant Ras, HIV and/or HER2 RNA, wild-type Ras, HIV and/or HER2 RNA, other proteins and/or RNAs involved in Ras, HIV and/or HER2 activity, compounds, metals, polymers, molecules and/or drugs that are targeted to Ras, HIV and/or
25 HER2 expressing cells etc., which, in turn, modulate the activity of the enzymatic nucleic acid molecule. In response to interaction with a predetermined signaling agent, the activity of the allosteric enzymatic nucleic acid molecule is activated or inhibited such that the expression of a particular target is selectively regulated, including down-regulated. The target can comprise wild-type Ras, HIV and/or HER2, mutant Ras, HIV and/or HER2, a component of Ras, HIV and/or HER2, and/or a predetermined cellular component that modulates Ras,
30 HIV and/or HER2 activity. For example, allosteric enzymatic nucleic acid molecules that are activated by interaction with a RNA encoding Ras, HIV and/or HER2 protein can be used as therapeutic agents *in vivo*. The presence of RNA encoding the Ras, HIV and/or HER2 protein activates the allosteric enzymatic nucleic acid molecule that subsequently cleaves the RNA encoding Ras, HIV and/or HER2 protein, resulting in the inhibition of Ras, HIV and/or

HER2 protein expression. In this manner, cells that express the Ras, HIV and/or HER2 protein are selectively targeted.

In another non-limiting example, an allozyme can be activated by a Ras, HIV and/or HER2 protein, peptide, or mutant polypeptide that causes the allozyme to inhibit the expression of Ras, HIV and/or HER2 gene, by, for example, cleaving RNA encoded by Ras, HIV and/or HER2 gene. In this non-limiting example, the allozyme acts as a decoy to inhibit the function of Ras, HIV and/or HER2 and also inhibit the expression of Ras, HIV and/or HER2 once activated by the Ras, HIV and/or HER2 protein.

Target sites

Targets for useful enzymatic nucleic acid molecules and antisense nucleic acids can be determined as disclosed in Draper *et al.*, WO 93/23569; Sullivan *et al.*, WO 93/23057; Thompson *et al.*, WO 94/02595; Draper *et al.*, WO 95/04818; McSwiggen *et al.*, US Patent No. 5,525,468, and hereby incorporated by reference herein in totality. Other examples include the following PCT applications, which concern inactivation of expression of disease-related genes: WO 95/23225, WO 95/13380, WO 94/02595, incorporated by reference herein. Rather than repeat the guidance provided in those documents here, below are provided specific non-limiting examples of such methods. Enzymatic nucleic acid molecules to such targets are designed as described in the above applications and synthesized to be tested *in vitro* and *in vivo*, as also described. The sequences of human K-Ras, H-Ras, HIV-1 and HER2 RNAs were screened for optimal enzymatic nucleic acid target sites using a computer-folding algorithm. Nucleic acid molecule binding/cleavage sites were identified. These sites are shown in the Tables (all sequences are 5' to 3' in the tables). The nucleotide base position is noted in the Tables as that site to be cleaved by the designated type of enzymatic nucleic acid molecule. Human sequences can be screened and enzymatic nucleic acid molecule and/or antisense thereafter designed, as discussed in Stinchcomb *et al.*, WO 95/23225. In addition, mouse targeted nucleic acid molecules can be used to test efficacy of action of the enzymatic nucleic acid molecule, siRNA and/or antisense prior to testing in humans.

In addition, enzymatic nucleic acid, siRNA, and antisense nucleic acid molecule binding/cleavage sites were identified. The nucleic acid molecules are individually analyzed by computer folding (Jaeger *et al.*, 1989 *Proc. Natl. Acad. Sci. USA*, 86, 7706) to assess whether the sequences fold into the appropriate secondary structure. Those nucleic acid molecules with unfavorable intramolecular interactions, such as between, for example the binding arms and the catalytic core of an enzymatic nucleic acid, are eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity.

Antisense, hammerhead, DNAzyme, NCH, amberzyme, zinzyme or G-Cleaver enzymatic nucleic acid molecule, siRNA, and antisense nucleic acid binding/cleavage sites were identified and were designed to anneal to various sites in the RNA target. The enzymatic nucleic acid binding arms or siRNA and antisense nucleic acid sequences are complementary to the target site sequences described above. The nucleic acid molecules are chemically synthesized. The method of synthesis used follows the procedure for normal DNA/RNA synthesis as described below and in Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19.

10 Synthesis of Nucleic acid Molecules

Synthesis of nucleic acids greater than 100 nucleotides in length can be difficult using automated methods, and the therapeutic cost of such molecules can be prohibitive. In this invention, small nucleic acid motifs ("small" refers to nucleic acid motifs less than about 100 nucleotides in length, preferably less than about 80 nucleotides in length, and more preferably less than about 50 nucleotides in length; *e.g.*, DNAzymes) are preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of RNA structure. Exemplary molecules of the instant invention are chemically synthesized as described herein, and others can similarly be synthesized.

Oligonucleotides (*e.g.*, DNAzymes, antisense) are synthesized using protocols known in the art as described in Caruthers *et al.*, 1992, *Methods in Enzymology* 211, 3-19, Thompson *et al.*, International PCT Publication No. WO 99/54459, Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684, Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, Brennan *et al.*, 1998, *Biotechnol Bioeng.*, 61, 33-45, and Brennan, US patent No. 6,001,311. All of these references are incorporated herein by reference. The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μ mol scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides and a 45 sec coupling step for 2'-deoxy nucleotides. Table I outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μ mol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μ L of 0.11 M = 6.6 μ mol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-ethyl tetrazole (60 μ L of 0.25 M = 15 μ mol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-

- bound 5'-hydroxyl. A 22-fold excess (40 μ L of 0.11 M = 4.4 μ mol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 μ L of 0.25 M = 10 μ mol) can be used in each coupling cycle of deoxy residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by
- 5 colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include; detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 16.9 mM I₂, 49 mM pyridine, 9% water in THF (PERSEPTIVE™).
- 10 Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.
- 15 Deprotection of the DNAzymes is performed as follows: the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to -20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H₂O/3:1:1, vortexed and the supernatant is then added to the first supernatant.
- 20 The combined supernatants, containing the oligoribonucleotide, are dried to a white powder.

- The method of synthesis used for RNA and chemically modified RNA or DNA, including certain enzymatic nucleic acid molecules and siRNA molecules, follows the procedure as described in Usman *et al.*, 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-
- 25 2684 Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μ mol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated
- 30 nucleotides. Table I outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μ mol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μ L of 0.11 M = 6.6 μ mol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 μ L of 0.25 M = 15 μ mol) can
- 35 be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-

hydroxyl. A 66-fold excess (120 μ L of 0.11 M = 13.2 μ mol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 μ L of 0.25 M = 30 μ mol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include; detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I₂, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide 0.05 M in acetonitrile) is used.

Deprotection of the RNA is performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to -20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H₂O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300 μ L of a solution of 1.5 mL *N*-methylpyrrolidinone, 750 μ L TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with 1.5 M NH₄HCO₃.

Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65 °C for 15 min. The vial is brought to r.t. TEA•3HF (0.1 mL) is added and the vial is heated at 65 °C for 15 min. The sample is cooled at -20 °C and then quenched with 1.5 M NH₄HCO₃.

For purification of the trityl-on oligomers, the quenched NH₄HCO₃ solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 0.5% TFA for 13 min. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile.

Inactive nucleic acid molecules or binding attenuated control (BAC) oligonucleotides can be synthesized by substituting one or more nucleotides in the nucleic acid molecule to inactivate the molecule and such molecules can serve as a negative control.

5 The average stepwise coupling yields are typically >98% (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted to be larger or smaller than the example described above including but not limited to 96 well format, all that is important is the ratio of chemicals used in the reaction.

10 Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example by ligation (Moore *et al.*, 1992, *Science* 256, 9923; Draper *et al.*, International PCT publication No. WO 93/23569; Shabarova *et al.*, 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.*, 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon *et al.*, 1997, *Bioconjugate Chem.* 8, 204).

15 The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.*, 1994, *Nucleic Acids Symp. Ser.* 31, 163). Enzymatic nucleic acid molecules are purified by gel electrophoresis using known methods or are purified by high pressure liquid chromatography (HPLC; See Wincott *et al.*, *Supra*, the totality of which is hereby
20 incorporated herein by reference) and are re-suspended in water.

The sequences of the nucleic acid molecules, including enzymatic nucleic acid molecules and antisense, that are chemically synthesized, are shown in the Tables herein. These sequences are representative only of many more such sequences where the enzymatic portion of the enzymatic nucleic acid molecule (all but the binding arms) is modified to affect
25 activity. For example, the enzymatic nucleic acid sequences listed in the Tables can be formed of deoxyribonucleotides or other nucleotides or non-nucleotides. Such enzymatic nucleic acid molecules with enzymatic activity are equivalent to the enzymatic nucleic acid molecules described specifically in the Tables.

Optimizing Activity of the Nucleic Acid Molecule of the Invention.

30 Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) that prevent their degradation by serum ribonucleases can increase their potency (see *e.g.*, Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken *et al.*, 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends*

in *Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. WO 93/15187; and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; and Burgin *et al.*, *supra*, all of which are hereby incorporated by reference in their entirety). All of the above references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications which enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

There are several examples of sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides can be modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS.* 17, 34; Usman *et al.*, 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). Sugar modification of nucleic acid molecules are also known to increase efficacy (see Eckstein *et al.*, International Publication PCT No. WO 92/07065; Perrault *et al.* *Nature*, 1990, 344, 565-568; Pieken *et al.* *Science*, 1991, 253, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.*, 1992, 17, 334-339; Usman *et al.* International Publication PCT No. WO 93/15187; Sproat, US Patent No. 5,334,711 and Beigelman *et al.*, 1995, *J. Biol. Chem.*, 270, 25702; Beigelman *et al.*, International PCT publication No. WO 97/26270; Beigelman *et al.*, US Patent No. 5,716,824; Usman *et al.*, US patent No. 5,627,053; Woolf *et al.*, International PCT Publication No. WO 98/13526; Thompson *et al.*, USSN 60/082,404 which was filed on April 20, 1998; Karpeisky *et al.*, 1998, *Tetrahedron Lett.*, 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic acid Sciences)*, 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.*, 67, 99-134; and Burlina *et al.*, 1997, *Bioorg. Med. Chem.*, 5, 1999-2010; all of the references are hereby incorporated in their totality by reference herein). The publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into enzymatic nucleic acid molecules without inhibiting catalysis. Similar modifications can be used as described herein to modify the nucleic acid molecules of the instant invention.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorothioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The

reduction in the concentration of these linkages can lower toxicity, resulting in increased efficacy and higher specificity of the therapeutic nucleic acid molecules.

5 Nucleic acid molecules having chemical modifications that maintain or enhance activity are provided. Such nucleic acid molecules are also generally more resistant to nucleases than unmodified nucleic acid molecules. Thus, the *in vitro* and/or *in vivo* activity should not be significantly lowered. Therapeutic nucleic acid molecules delivered exogenously are optimally stable within cells until translation of the target RNA has been inhibited long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days, depending upon the disease state. Nucleic acid molecules are preferably
10 resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of RNA and DNA (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19 (incorporated by reference herein)) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

15 In one embodiment, nucleic acid molecules of the invention include one or more G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein modifications result in the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, *J. Am. Chem. Soc.*, 120, 8531-8532. A single G-clamp analog substitution within an
20 oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid molecules of the invention can enable both enhanced affinity and specificity to nucleic acid targets.

In another embodiment, the invention features conjugates and/or complexes of nucleic
25 acid molecules targeting Ras genes such as K-Ras, H-Ras, and/or N-Ras. Compositions and conjugates are used to facilitate delivery of molecules into a biological system, such as cells. The conjugates provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the invention. The present
30 invention encompasses the design and synthesis of novel agents for the delivery of molecules, including but not limited to, small molecules, lipids, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed to be used

either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, US 5,854,038). Conjugates of
5 the molecules described herein can be attached to biologically active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

The term "biodegradable nucleic acid linker molecule" as used herein, refers to a nucleic acid molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule. The stability of the
10 biodegradable nucleic acid linker molecule can be modulated by using various combinations of ribonucleotides, deoxyribonucleotides, and chemically modified nucleotides, for example 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2,
15 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

20 The term "biodegradable" as used herein, refers to degradation in a biological system, for example, enzymatic degradation or chemical degradation.

The term "biologically active molecule" as used herein, refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active molecules contemplated by the instant invention
25 include therapeutically active molecules such as antibodies, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siRNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of
30 modulating the pharmacokinetics and/or pharmacodynamics of other biologically active molecules, for example lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus

containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

Use of the nucleic acid-based molecules of the invention can lead to better treatment of the disease progression by affording the possibility of combination therapies (e.g., multiple antisense or enzymatic nucleic acid molecules targeted to different genes, nucleic acid molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations of molecules (including different motifs) and/or other chemical or biological molecules). The treatment of subjects with nucleic acid molecules can also include combinations of different types of nucleic acid molecules.

10 In the case that down-regulation of the target is desired, therapeutic nucleic acid molecules (e.g., DNAzymes) delivered exogenously are optimally stable within cells until translation of the target RNA has been inhibited long enough to reduce the levels of the targeted protein. This period of time varies between hours to days depending upon the disease state. These nucleic acid molecules should be resistant to nucleases in order to
15 function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid molecules described in the instant invention and others known in the art have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

In another embodiment, nucleic acid catalysts having chemical modifications that
20 maintain or enhance enzymatic activity are provided. Such nucleic acids are also generally more resistant to nucleases than unmodified nucleic acid. Thus, the *in vitro* and/or *in vivo* the activity of the nucleic acid should not be significantly lowered. As exemplified herein, such enzymatic nucleic acids are useful for *in vitro* and/or *in vivo* techniques even if activity over all is reduced 10 fold (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). Such enzymatic
25 nucleic acids herein are said to "maintain" the enzymatic activity of an all RNA ribozyme or all DNA DNAzyme.

In another aspect the nucleic acid molecules comprise a 5' and/or a 3'-cap structure.

By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Wincott *et al.*, WO 97/26270,
30 incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and can help in delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap) or at the 3'-terminus (3'-cap) or can be present on both termini. In non-limiting examples, the 5'-cap includes inverted abasic

residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-erythrofuransyl) nucleotide, 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuransyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety (for more details see Wincott *et al.*, International PCT publication No. WO 97/26270, incorporated by reference herein).

In another embodiment, the 3'-cap includes, for example 4',5'-methylene nucleotide; 1-(beta-D-erythrofuransyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-aminoalkyl phosphate; 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuransyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine.

The term "alkyl" as used herein refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain "isoalkyl", and cyclic alkyl groups. The term "alkyl" also comprises alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably it is a lower alkyl of from about 1 to 7 carbons, more preferably about 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) preferably comprise hydroxy, oxy, thio, amino, nitro, cyano, alkoxy,

alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, silyl, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups. The term "alkyl" also includes alkenyl groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has about 2 to 12 carbons. More preferably it is a lower alkenyl of from about 2 to 7 carbons, more preferably about 2 to 4 carbons. The alkenyl group can be substituted or unsubstituted. When substituted the substituted group(s) preferably comprise hydroxy, oxy, thio, amino, nitro, cyano, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, silyl, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups.

The term "alkyl" also includes alkynyl groups containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has about 2 to 12 carbons. More preferably it is a lower alkynyl of from about 2 to 7 carbons, more preferably about 2 to 4 carbons. The alkynyl group can be substituted or unsubstituted. When substituted the substituted group(s) preferably comprise hydroxy, oxy, thio, amino, nitro, cyano, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, silyl, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups. Alkyl groups or moieties of the invention can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from about 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

The term "alkoxyalkyl" as used herein refers to an alkyl-O-alkyl ether, for example, methoxyethyl or ethoxymethyl.

The term "alkyl-thio-alkyl" as used herein refers to an alkyl-S-alkyl thioether, for example, methylthiomethyl or methylthioethyl.

5 The term "amino" as used herein refers to a nitrogen containing group as is known in the art derived from ammonia by the replacement of one or more hydrogen radicals by organic radicals. For example, the terms "aminoacyl" and "aminoalkyl" refer to specific N-substituted organic radicals with acyl and alkyl substituent groups respectively.

The term "amination" as used herein refers to a process in which an amino group or substituted amine is introduced into an organic molecule.

10 The term "exocyclic amine protecting moiety" as used herein refers to a nucleobase amino protecting group compatible with oligonucleotide synthesis, for example, an acyl or amide group.

The term "alkenyl" as used herein refers to a straight or branched hydrocarbon of a designed number of carbon atoms containing at least one carbon-carbon double bond. Examples of "alkenyl" include vinyl, allyl, and 2-methyl-3-heptene.

15 The term "alkoxy" as used herein refers to an alkyl group of indicated number of carbon atoms attached to the parent molecular moiety through an oxygen bridge. Examples of alkoxy groups include, for example, methoxy, ethoxy, propoxy and isopropoxy.

20 The term "alkynyl" as used herein refers to a straight or branched hydrocarbon of a designed number of carbon atoms containing at least one carbon-carbon triple bond. Examples of "alkynyl" include propargyl, propyne, and 3-hexyne.

25 The term "aryl" as used herein refers to an aromatic hydrocarbon ring system containing at least one aromatic ring. The aromatic ring can optionally be fused or otherwise attached to other aromatic hydrocarbon rings or non-aromatic hydrocarbon rings. Examples of aryl groups include, for example, phenyl, naphthyl, 1,2,3,4-tetrahydronaphthalene and biphenyl. Preferred examples of aryl groups include phenyl and naphthyl.

The term "cycloalkenyl" as used herein refers to a C3-C8 cyclic hydrocarbon containing at least one carbon-carbon double bond. Examples of cycloalkenyl include cyclopropenyl, cyclobutenyl, cyclopentenyl, cyclopentadiene, cyclohexenyl, 1,3-cyclohexadiene, cycloheptenyl, cycloheptatrienyl, and cyclooctenyl.

The term "cycloalkyl" as used herein refers to a C3-C8 cyclic hydrocarbon. Examples of cycloalkyl include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl.

5 The term "cycloalkylalkyl," as used herein, refers to a C3-C7 cycloalkyl group attached to the parent molecular moiety through an alkyl group, as defined above. Examples of cycloalkylalkyl groups include cyclopropylmethyl and cyclopentylethyl.

The terms "halogen" or "halo" as used herein refers to indicate fluorine, chlorine, bromine, and iodine.

10 The term "heterocycloalkyl," as used herein refers to a non-aromatic ring system containing at least one heteroatom selected from nitrogen, oxygen, and sulfur. The heterocycloalkyl ring can be optionally fused to or otherwise attached to other heterocycloalkyl rings and/or non-aromatic hydrocarbon rings. Preferred heterocycloalkyl groups have from 3 to 7 members. Examples of heterocycloalkyl groups include, for example, piperazine, morpholine, piperidine, tetrahydrofuran, pyrrolidine, and pyrazole.
15 Preferred heterocycloalkyl groups include piperidinyl, piperazinyl, morpholinyl, and pyrrolidinyl.

The term "heteroaryl" as used herein refers to an aromatic ring system containing at least one heteroatom selected from nitrogen, oxygen, and sulfur. The heteroaryl ring can be fused or otherwise attached to one or more heteroaryl rings, aromatic or non-aromatic hydrocarbon rings or heterocycloalkyl rings. Examples of heteroaryl groups include, for example, pyridine, furan, thiophene, 5,6,7,8-tetrahydroisoquinoline and pyrimidine. Preferred examples of heteroaryl groups include thienyl, benzothienyl, pyridyl, quinolyl, pyrazinyl, pyrimidyl, imidazolyl, benzimidazolyl, furanyl, benzofuranyl, thiazolyl, benzothiazolyl, isoxazolyl, oxadiazolyl, isothiazolyl, benzisothiazolyl, triazolyl, tetrazolyl, pyrrolyl, indolyl,
20 pyrazolyl, and benzopyrazolyl.
25

The term "C1-C6 hydrocarbyl" as used herein refers to straight, branched, or cyclic alkyl groups having 1-6 carbon atoms, optionally containing one or more carbon-carbon double or triple bonds. Examples of hydrocarbyl groups include, for example, methyl, ethyl, propyl, isopropyl, n-butyl, sec-butyl, tert-butyl, pentyl, 2-pentyl, isopentyl, neopentyl, hexyl,
30 2-hexyl, 3-hexyl, 3-methylpentyl, vinyl, 2-pentene, cyclopropylmethyl, cyclopropyl, cyclohexylmethyl, cyclohexyl and propargyl. When reference is made herein to C1-C6 hydrocarbyl containing one or two double or triple bonds it is understood that at least two

carbons are present in the alkyl for one double or triple bond, and at least four carbons for two double or triple bonds.

By "nucleotide" is meant a heterocyclic nitrogenous base in N-glycosidic linkage with a phosphorylated sugar. Nucleotides are recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra* all are hereby incorporated by reference herein. There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, 1994, Nucleic Acids Res. 22, 2183. Some of the non-limiting examples of chemically modified and other natural nucleic acid bases that can be introduced into nucleic acids include, for example, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, queosine, 2-thiouridine, 4-thiouridine, wybutosine, wybutoxosine, 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 5'-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, beta-D-galactosylqueosine, 1-methyladenosine, 1-methylinosine, 2,2-dimethylguanosine, 3-methylcytidine, 2-methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-methylcarbonylmethyluridine, 5-methoxyuridine, 5-methyl-2-thiouridine, 2-methylthio-N6-isopentenyladenosine, beta-D-mannosylqueosine, uridine-5-oxyacetic acid, 2-thiocytidine, threonine derivatives and others (Burgin *et al.*, 1996, Biochemistry, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases can be used at any position, for example, within the catalytic core of an enzymatic nucleic acid molecule and/or in the substrate-binding regions of the nucleic acid molecule.

By "nucleoside" is meant a heterocyclic nitrogenous base in N-glycosidic linkage with a sugar. Nucleosides are recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a

nucleoside sugar moiety. Nucleosides generally comprise a base and sugar group. The nucleosides can be unmodified or modified at the sugar, and/or base moiety (also referred to interchangeably as nucleoside analogs, modified nucleosides, non-natural nucleosides, non-standard nucleosides and other; see for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra* all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of chemically modified and other natural nucleic acid bases that can be introduced into nucleic acids include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, quinosine, 2-thiouridine, 4-thiouridine, wybutosine, wybutoxosine, 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 5'-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, beta-D-galactosylquosine, 1-methyladenosine, 1-methylinosine, 2,2-dimethylguanosine, 3-methylcytidine, 2-methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-methylcarbonylmethyluridine, 5-methoxyuridine, 5-methyl-2-thiouridine, 2-methylthio-N6-isopentenyladenosine, beta-D-mannosylquosine, uridine-5-oxyacetic acid, 2-thiocytidine, threonine derivatives and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleoside bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases can be used at any position, for example, within the catalytic core of an enzymatic nucleic acid molecule and/or in the substrate-binding regions of the nucleic acid molecule.

In one embodiment, the invention features modified enzymatic nucleic acid molecules with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications see Hunziker and Leumann, 1995, *Nucleic Acid Analogues: Synthesis and Properties*, in *Modern Synthetic Methods*, VCH, 331-417, and Mesmaeker *et al.*, 1994, *Novel Backbone Replacements for Oligonucleotides*, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39. These references are hereby incorporated by reference herein.

By "abasic" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, for example a 3',3'-linked or 5',5'-linked deoxyabasic ribose derivative (for more details see Wincott *et al.*, International PCT publication No. WO 97/26270).

- 5 By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine, thymine, uracil joined to the 1' carbon of β -D-ribo-furanose.

By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate.

- 10 In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH₂ or 2'-O- NH₂, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein *et al.*, U.S. Patent 5,672,695 and Matulic-Adamic *et al.*, WO 98/28317, respectively, which are both incorporated by reference in their entireties.

- 15 Various modifications to nucleic acid (*e.g.*, DNAzyme) structure can be made to enhance the utility of these molecules. For example, such modifications can enhance shelf-life, half-life *in vitro*, stability, and ease of introduction of such oligonucleotides to the target site, including *e.g.*, enhancing penetration of cellular membranes and conferring the ability to recognize and bind to targeted cells.

- 20 Use of these molecules can lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple enzymatic nucleic acid molecules targeted to different genes, enzymatic nucleic acid molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations of enzymatic nucleic acid molecules (including different enzymatic nucleic acid molecule motifs) and/or other chemical or biological molecules). The treatment of subjects with nucleic acid molecules can also include combinations of different types of nucleic acid molecules. Therapies can be devised which include a mixture of enzymatic nucleic acid molecules (including different enzymatic nucleic acid molecule motifs), antisense and/or 2-5A chimera molecules to one or more targets to alleviate symptoms of a disease.

Administration of Nucleic Acid Molecules

- 30 Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.*, 1992, *Trends Cell Bio.*, 2, 139; and *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995, which are both incorporated herein by reference. Sullivan *et al.*, PCT WO

94/02595, further describes the general methods for delivery of enzymatic RNA molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Other routes of delivery include, but are not limited to oral (tablet or pill form) and/or intrathecal delivery (Gold, 1997, *Neuroscience*, 76, 1153-1158). Other approaches include the use of various transport and carrier systems, for example though the use of conjugates and biodegradable polymers. For a comprehensive review on drug delivery strategies including CNS delivery, see Ho *et al.*, 1999, *Curr. Opin. Mol. Ther.*, 1, 336-343 and Jain, *Drug Delivery Systems: Technologies and Commercial Opportunities*, Decision Resources, 1998 and Groothuis *et al.*, 1997, *J. NeuroVirol.*, 3, 387-400. More detailed descriptions of nucleic acid delivery and administration are provided in Sullivan *et al.*, supra, Draper *et al.*, PCT WO93/23569, Beigelman *et al.*, PCT WO99/05094, and Klimuk *et al.*, PCT WO99/04819, all of which have been incorporated by reference herein.

The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a subject.

The negatively charged polynucleotides of the invention can be administered (e.g., RNA, DNA or protein) and introduced into a subject by any standard means described herein and known in the art, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention can also be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions; suspensions for injectable administration; and the other compositions known in the art.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, e.g., acid addition salts, for example, salts of hydrochloric; hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, e.g., systemic administration, into a cell or subject,

preferably a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (*i.e.*, a cell to which the negatively charged polymer is desired to be delivered to). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms which prevent the composition or formulation from exerting its effect.

By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without limitations: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes expose the desired negatively charged polymers, *e.g.*, nucleic acids, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach can provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as cancer cells.

By pharmaceutically acceptable formulation is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: PEG conjugated nucleic acids, phospholipid conjugated nucleic acids, nucleic acids containing lipophilic moieties, phosphorothioates, P-glycoprotein inhibitors (such as Pluronic P85) which can enhance entry of drugs into various tissues, for example the CNS (Joliet-Riant and Tillement, 1999, *Fundam. Clin. Pharmacol.*, 13, 16-26); biodegradable polymers, such as poly (DL-lactide-co-glycolide) microspheres for sustained release delivery after implantation (Emerich, DF *et al*, 1999, *Cell Transplant*, 8, 47-58) Alkermes, Inc. Cambridge, MA; and loaded nanoparticles, such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (*Prog Neuropsychopharmacol Biol Psychiatry*, 23, 941-949, 1999). Other non-limiting examples of delivery strategies, including CNS delivery of the nucleic acid molecules of the instant

invention include material described in Boado *et al.*, 1998, *J. Pharm. Sci.*, 87, 1308-1315; Tyler *et al.*, 1999, *FEBS Lett.*, 421, 280-284; Pardridge *et al.*, 1995, *PNAS USA.*, 92, 5592-5596; Boado, 1995, *Adv. Drug Delivery Rev.*, 15, 73-107; Aldrian-Herrada *et al.*, 1998, *Nucleic Acids Res.*, 26, 4910-4916; and Tyler *et al.*, 1999, *PNAS USA.*, 96, 7053-7058. All
5 these references are hereby incorporated herein by reference.

The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). Nucleic acid molecules of the invention can also comprise covalently attached PEG molecules of various molecular weights. These formulations offer a
10 method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al. Chem. Rev.* 1995, 95, 2601-2627; Ishiwata *et al., Chem. Pharm. Bull.* 1995, 43, 1005-1011). Such liposomes have been shown to accumulate
15 selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al., Science* 1995, 267, 1275-1276; Oku *et al., 1995, Biochim. Biophys. Acta*, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes, which are known to accumulate in tissues of the MPS (Liu *et al., J. Biol. Chem.*
20 1995, 42, 24864-24870; Choi *et al., International PCT Publication No. WO 96/10391*; Ansell *et al., International PCT Publication No. WO 96/10390*; Holland *et al., International PCT Publication No. WO 96/10392*; all of which are incorporated by reference herein). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in
25 metabolically aggressive MPS tissues such as the liver and spleen. All of these references are incorporated by reference herein.

The present invention also includes compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic
30 use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors which those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

The nucleic acid molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (e.g., intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia, and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate can be employed.

Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

- 5 Aqueous suspensions contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example, sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example, ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.
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- 20 Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid.

- 25 Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

- 30 Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or

partial esters derived from fatty acids and hexitol, anhydrides, for example, sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

5 Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing
10 or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed
15 as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The nucleic acid molecules of the invention can also be administered in the form of suppositories, *e.g.*, for rectal administration of the drug. These compositions can be prepared
20 by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

Nucleic acid molecules of the invention can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended
25 or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per patient or subject per day). The amount of active ingredient that can be
30 combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient.

It is understood that the specific dose level for any particular patient or subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

- 5 For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the composition as a premix for addition to the feed or drinking water.
- 10 The nucleic acid molecules of the present invention can also be administered to a patient or subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

- 15 In another aspect of the invention, nucleic acid molecules of the present invention are preferably expressed from transcription units (see for example Couture *et al.*, 1996, *TIG.*, 12, 510, Skillem *et al.*, International PCT Publication No. WO 00/22113, Conrad, International PCT Publication No. WO 00/22114, and Conrad, US 6,054,299) inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Enzymatic nucleic acid expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the nucleic acid molecules are delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the nucleic acid molecule binds to the target mRNA. Delivery of nucleic acid molecule expressing vectors can be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from the subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell (for a review see Couture *et al.*, 1996, *TIG.*, 12, 510).
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- 30 One aspect of the invention features an expression vector comprising a nucleic acid sequence encoding at least one of the nucleic acid molecules of the instant invention. The nucleic acid sequence encoding the nucleic acid molecule of the instant invention is operably linked in a manner that allows expression of that nucleic acid molecule.

Another aspect the invention features an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecules of the invention, in a manner which allows expression of that nucleic acid molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; c) a nucleic acid sequence encoding at least one said nucleic acid molecule; and wherein said sequence is operably linked to said initiation region and said termination region, in a manner that allows expression and/or delivery of said nucleic acid molecule.

In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; d) a nucleic acid sequence encoding at least one said nucleic acid molecule, wherein said sequence is operably linked to the 3'-end of said open reading frame; and wherein said sequence is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In yet another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) a nucleic acid sequence encoding at least one said nucleic acid molecule; and wherein said sequence is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading frame; e) a nucleic acid sequence encoding at least one said nucleic acid molecule, wherein said sequence is operably linked to the 3'-end of said open reading frame; and wherein said sequence is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

Examples

The following are non-limiting examples showing the selection, isolation, synthesis and activity of nucleic acids of the instant invention.

Example 1: Identification of Potential Target Sites in Human Ras RNA

The sequence of human Ras genes were screened for accessible sites using a computer-folding algorithm. Regions of the RNA that do not form secondary folding structures and contain potential enzymatic nucleic acid molecule and/or antisense binding/cleavage sites

were identified. The sequences of K-Ras and H-Ras binding/cleavage sites are shown in Tables II and III.

Example 2: Selection of Enzymatic Nucleic Acid Cleavage Sites in Human Ras RNA

Enzymatic nucleic acid molecule target sites were chosen by analyzing sequences of Human K-Ras and H-Ras (for example, Genbank accession Nos: NM_004985 and NM_005343 respectively) and prioritizing the sites on the basis of folding. Enzymatic nucleic acid molecules were designed that can bind each target and were individually analyzed by computer folding (Christoffersen *et al.*, 1994 *J. Mol. Struc. Theochem*, 311, 273; Jaeger *et al.*, 1989, *Proc. Natl. Acad. Sci. USA*, 86, 7706) to assess whether the enzymatic nucleic acid molecule sequences fold into the appropriate secondary structure. Those enzymatic nucleic acid molecules with unfavorable intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. As noted below, varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 bases on each arm are able to bind to, or otherwise interact with, the target RNA.

15 Example 3: Chemical Synthesis and Purification of Enzymatic Nucleic Acid Molecules for Efficient Cleavage and/or blocking of Ras RNA

DNAzyme molecules are designed to anneal to various sites in the RNA message. The binding arms of the DNAzyme molecules are complementary to the target site sequences described above. The DNAzymes were chemically synthesized. The method of synthesis used followed the procedure for nucleic acid synthesis as described herein and in Usman *et al.*, (1987 *J. Am. Chem. Soc.*, 109, 7845), Scaringe *et al.*, (1990 *Nucleic Acids Res.*, 18, 5433) and Wincott *et al.*, *supra*, and made use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. The average stepwise coupling yields were typically >98%. The sequences of the chemically synthesized DNAzyme molecules used in this study are shown below in Tables II and III.

Example 4: DNAzyme Cleavage of Ras RNA Target *in vitro*

DNAzymes targeted to the human K-Ras and H-Ras RNA are designed and synthesized as described above. These enzymatic nucleic acid molecules can be tested for cleavage activity *in vitro*, for example, using the following procedure. The target sequences and the nucleotide location within the K-Ras and H-Ras RNA are given in Tables II and III respectively.

Cleavage Reactions:

DNAzymes and substrates were synthesized in 96-well format using 0.2 μ mol scale. Substrates were 5'-³²P labeled and gel purified using 7.5% polyacrylamide gels, and eluting into water. Assays were done by combining trace substrate with 500nM DNAzyme or greater, and initiated by adding final concentrations of 40mM Mg⁺², and 50mM Tris-Cl pH 8.0. For each DNAzyme/substrate combination a control reaction was done to ensure cleavage was not the result of non-specific substrate degradation. A single three hour time point was taken and run on a 15% polyacrylamide gel to asses cleavage activity. Gels were dried and scanned using a Molecular Dynamics Phosphorimager and quantified using Molecular Dynamics ImageQuant software. Percent cleaved was determined by dividing values for cleaved substrate bands by full-length (uncleaved) values plus cleaved values and multiplying by 100 (%cleaved=[C/(U+C)]*100).

Example 5: DNAzyme Cleavage of Ras RNA Target *in vivo*

Cell Culture

Wickstrom, 2001, *Mol. Biotechnol.*, 18, 35-35, describes a cell culture system in which antisense oligonucleotides targeting H-Ras were studied in transformed mouse cells that form solid tumors. Treatment of cells with antisense targeting H-Ras resulted in the sequence specific and dose dependent inhibition of H-Ras expression. In this study, it was determined that antisense targeting the first intron region of H-Ras were more effective than antisense targeting the initiation codon region.

Kita *et al.*, 1999, *Int. J. Cancer*, 80, 553-558, describes the growth inhibition of human pancreatic cancer cell lines by antisense oligonucleotides specific to mutated K-Ras genes. Antisense oligonucleotides were transfected to the transformed cells using liposomes. Cellular proliferation, K-Ras mRNA expression, and K-Ras protein synthesis were all evaluated as endpoints. Sato *et al.*, 2000, *Cancer Lett.*, 155, 153-161, describes another human pancreatic cancer cell line, HOR-P1, that is characterized by high angiogenic activity and metastatic potential. Genetic and molecular analysis of this cell line revealed both increased telomerase activity and a mutation in the K-Ras oncogene.

A variety of endpoints have been used in cell culture models to look at Ras-mediated effects after treatment with anti-Ras agents. Phenotypic endpoints include inhibition of cell proliferation, RNA expression, and reduction of Ras protein expression. Because Ras oncogene mutations are directly associated with increased proliferation of cetain tumor cells,

a proliferation endpoint for cell culture assays is preferably used as the primary screen. There are several methods by which this endpoint can be measured. Following treatment of cells with DNazymes, cells are allowed to grow (typically 5 days) after which either the cell viability, the incorporation of [³H] thymidine into cellular DNA and/or the cell density can be measured. The assay of cell density is done in a 96-well format using commercially available fluorescent nucleic acid stains (such as Syto® 13 or CyQuant®). As a secondary, confirmatory endpoint a DNzyme-mediated decrease in the level of Ras protein expression is evaluated using a Ras-specific ELISA.

Animal Models

Evaluating the efficacy of anti-Ras agents in animal models is an important prerequisite to human clinical trials. As in cell culture models, the most Ras sensitive mouse tumor xenografts are those derived from cancer cells that express mutant Ras proteins. Nude mice bearing H-Ras transformed bladder cancer cell xenografts were sensitive to an anti-Ras antisense nucleic acid, resulting in an 80% inhibition of tumor growth after a 31 day treatment period (Wickstrom, 2001, *Mol. Biotechnol.*, 18, 35-35). Zhang *et al.*, 2000, *Gene Ther.*, 7, 2041, describes an anti-K-Ras ribozyme adenoviral vector (KRbz-ADV) targeting a K-Ras mutant (K-Ras codon 12 GGT to GTT; H441 and H1725 cells respectively). Non-small cell lung cancer cells (NSCLC H441 and H1725 cells) that express the mutant K-Ras protein were used in nude mouse xenografts compared to NSCLC H1650 cells that lack the relevant mutation. Pre-treatment with KRbz-ADV completely abrogated engraftment of both H441 and H1725 cells and compared to 100% engraftment and tumor growth in animals that received untreated tumor cells or a control vector. The above studies provide proof that inhibition of Ras expression by anti-Ras agents causes inhibition of tumor growth in animals. Anti-Ras DNazymes chosen from *in vitro* assays are further tested in similar mouse xenograft models. Active DNazymes are subsequently tested in combination with standard chemotherapies.

Indications

Particular degenerative and disease states that are associated with Ras expression modulation include but are not limited to cancer, for example lung cancer, colorectal cancer, bladder cancer, pancreatic cancer, breast cancer, prostate cancer and/or any other diseases or conditions that are related to or will respond to the levels of Ras in a cell or tissue, alone or in combination with other therapies.

The present body of knowledge in Ras research indicates the need for methods to assay Ras activity and for compounds that can regulate Ras expression for research, diagnostic, and therapeutic use.

5 The use of monoclonal antibodies, chemotherapy, radiation therapy, and analgesics, are all non-limiting examples of methods that can be combined with or used in conjunction with the nucleic acid molecules (e.g. DNazymes) of the instant invention. Common chemotherapies that can be combined with nucleic acid molecules of the instant invention include various combinations of cytotoxic drugs to kill cancer cells. These drugs include but are not limited to paclitaxel (Taxol), docetaxel, cisplatin, methotrexate, cyclophosphamide, 10 doxorubin, fluorouracil carboplatin, edatrexate, gemcitabine, vinorelbine etc. Those skilled in the art will recognize that other drug compounds and therapies can be similarly be readily combined with the nucleic acid molecules of the instant invention (e.g. DNzyme molecules) are hence within the scope of the instant invention.

Diagnostic uses

15 The nucleic acid molecules of this invention (e.g., enzymatic nucleic acid molecules) are used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of Ras RNA in a cell. The close relationship between enzymatic nucleic acid molecule activity and the structure of the target RNA allows the detection of mutations in any region of the molecule that alters the base-pairing and three-dimensional structure of the target RNA. Using multiple enzymatic nucleic acid molecules described in this invention, 20 one maps nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with enzymatic nucleic acid molecules are used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets are defined as important 25 mediators of the disease. These experiments lead to better treatment of the disease progression by affording the possibility of combinational therapies (e.g., multiple enzymatic nucleic acid molecules targeted to different genes, enzymatic nucleic acid molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations of enzymatic nucleic acid molecules and/or other chemical or biological molecules). Other *in vitro* 30 uses of enzymatic nucleic acid molecules of this invention are known in the art, and include detection of the presence of mRNAs associated with Ras-related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with an enzymatic nucleic acid molecule using standard methodology.

In a specific example, enzymatic nucleic acid molecules that cleave only wild-type or mutant forms of the target RNA are used for the assay. The first enzymatic nucleic acid molecule is used to identify wild-type RNA present in the sample and the second enzymatic nucleic acid molecule is used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both enzymatic nucleic acid molecules to demonstrate the relative enzymatic nucleic acid molecule efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus each analysis requires two enzymatic nucleic acid molecules, two substrates and one unknown sample which is combined into six reactions. The presence of cleavage products is determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (*i.e.*, Ras) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the initial diagnosis. Higher mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared qualitatively or quantitatively. The use of enzymatic nucleic acid molecules in diagnostic applications contemplated by the instant invention is described, for example, in George *et al.*, US Patent Nos. 5,834,186 and 5,741,679, Shih *et al.*, US Patent No. 5,589,332, Nathan *et al.*, US Patent No 5,871,914, Nathan and Ellington, International PCT publication No. WO 00/24931, Breaker *et al.*, International PCT Publication Nos. WO 00/26226 and 98/27104, and Sullenger *et al.*, International PCT publication No. WO 99/29842.

Example 6: Identification of Potential Target Sites in Human HIV RNA

The sequence of human HIV genes are screened for accessible sites using a computer-folding algorithm. Regions of the RNA that do not form secondary folding structures and contained potential enzymatic nucleic acid molecule and/or antisense binding/cleavage sites are identified. The sequences of these binding/cleavage sites are shown in Tables VI to XI.

Example 6: Selection of Enzymatic Nucleic Acid Cleavage Sites in Human HIV RNA

Enzymatic nucleic acid molecule target sites were chosen by analyzing sequences of Human HIV (Genbank accession No: NM_005228) and prioritizing the sites on the basis of

folding. Enzymatic nucleic acid molecules were designed that can bind each target and are individually analyzed by computer folding (Christoffersen *et al.*, 1994 *J. Mol. Struct. Theochem*, 311, 273; Jaeger *et al.*, 1989, *Proc. Natl. Acad. Sci. USA*, 86, 7706) to assess whether the enzymatic nucleic acid molecule sequences fold into the appropriate secondary structure. Those enzymatic nucleic acid molecules with unfavorable intramolecular interactions between the binding arms and the catalytic core were eliminated from consideration. As noted below, varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 bases on each arm are able to bind to, or otherwise interact with, the target RNA.

10 Example 8: Chemical Synthesis and Purification of Ribozymes and Antisense for Efficient Cleavage and/or blocking of HIV Activity

Enzymatic nucleic acid molecules and antisense constructs are designed to anneal to various sites in the RNA message. The binding arms of the enzymatic nucleic acid molecules are complementary to the target site sequences described above, while the antisense constructs are fully complementary to the target site sequences described above. The enzymatic nucleic acid molecules and antisense constructs were chemically synthesized. The method of synthesis used followed the procedure for normal RNA synthesis as described above and in Usman *et al.*, (1987 *J. Am. Chem. Soc.*, 109, 7845), Scaringe *et al.*, (1990 *Nucleic Acids Res.*, 18, 5433) and Wincott *et al.*, *supra*, and made use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. The average stepwise coupling yields were typically >98%.

Enzymatic nucleic acid molecules and antisense constructs are also synthesized from DNA templates using bacteriophage T7 RNA polymerase (Milligan and Uhlenbeck, 1989, *Methods Enzymol.* 180, 51). Enzymatic nucleic acid molecules and antisense constructs are purified by gel electrophoresis using general methods or are purified by high pressure liquid chromatography (HPLC; See Wincott *et al.*, *supra*; the totality of which is hereby incorporated herein by reference) and are resuspended in water. The sequences of the chemically synthesized enzymatic nucleic acid molecules used in this study are shown below in Table XI. The sequences of the chemically synthesized antisense constructs used in this study are complementary sequences to the Substrate sequences shown below as in Tables VI to XI.

Example 8: Enzymatic nucleic acid molecule Cleavage of HIV RNA Target *in vitro*

Enzymatic nucleic acid molecules targeted to the human HIV RNA are designed and synthesized as described above. These enzymatic nucleic acid molecules are tested for cleavage activity *in vitro*, for example, using the following procedure. The target sequences and the nucleotide location within the HIV RNA are given in Tables VI to XI.

- 5 *Cleavage Reactions:* Full-length or partially full-length, internally-labeled target RNA for enzymatic nucleic acid molecule cleavage assay is prepared by *in vitro* transcription in the presence of [α - 32 P] CTP, passed over a G 50 Sephadex column by spin chromatography and used as substrate RNA without further purification. Alternately, substrates are 5'- 32 P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed by pre-warming a 2X
- 10 concentration of purified enzymatic nucleic acid molecule in enzymatic nucleic acid molecule cleavage buffer (50 mM Tris-HCl, pH 7.5 at 37°C, 10 mM MgCl₂) and the cleavage reaction was initiated by adding the 2X enzymatic nucleic acid molecule mix to an equal volume of substrate RNA (maximum of 1-5 nM) that was also pre-warmed in cleavage buffer. As an
- 15 initial screen, assays are carried out for 1 hour at 37°C using a final concentration of either 40 nM or 1 mM enzymatic nucleic acid molecule, *i.e.*, enzymatic nucleic acid molecule excess. The reaction is quenched by the addition of an equal volume of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol after which the sample is heated to 95°C for 2 minutes, quick chilled and loaded onto a denaturing polyacrylamide gel. Substrate RNA and the specific RNA cleavage products generated by enzymatic nucleic acid
- 20 molecule cleavage are visualized on an autoradiograph of the gel. The percentage of cleavage is determined by Phosphor Imager[®] quantitation of bands representing the intact substrate and the cleavage products.

Indications

- 25 Particular degenerative and disease states that can be associated with HIV expression modulation include but are not limited to acquired immunodeficiency disease (AIDS) and related diseases and conditions, including but not limited to Kaposi's sarcoma, lymphoma, cervical cancer, squamous cell carcinoma, cardiac myopathy, rheumatic diseases, and opportunistic infection, for example Pneumocystis carinii, Cytomegalovirus, Herpes simplex, Mycobacteria, Cryptococcus, Toxoplasma, Progressive multifocal leucoencephalopathy
- 30 (Papovavirus), Mycobacteria, Aspergillus, Cryptococcus, Candida, Cryptosporidium, Isospora belli, Microsporidia and any other diseases or conditions that are related to or will respond to the levels of HIV in a cell or tissue, alone or in combination with other therapies

The present body of knowledge in HIV research indicates the need for methods to assay HIV activity and for compounds that can regulate HIV expression for research, diagnostic, and therapeutic use.

5 The use of antiviral compounds, monoclonal antibodies, chemotherapy, radiation
therapy, analgesics, and/or anti-inflammatory compounds, are all non-limiting examples of a
methods that can be combined with or used in conjunction with the nucleic acid molecules
(e.g. ribozymes and antisense molecules) of the instant invention. Examples of antiviral
compounds that can be used in conjunction with the nucleic acid molecules of the invention
include but are not limited to AZT (also known as zidovudine or ZDV), ddC (zalcitabine), ddI
10 (dideoxyinosine), d4T (stavudine), and 3TC (lamivudine) Ribavirin, delvaridine (Rescriptor),
nevirapine (Viramune), efavirenz (Sustiva), ritonavir (Norvir), saquinavir (Invirase),
indinavir (Crixivan), amprenavir (Agenerase), nelfinavir (Viracept), and/or lopinavir
(Kaletra). Common chemotherapies that can be combined with nucleic acid molecules of the
instant invention include various combinations of cytotoxic drugs to kill cancer cells. These
15 drugs include but are not limited to paclitaxel (Taxol), docetaxel, cisplatin, methotrexate,
cyclophosphamide, doxorubin, fluorouracil carboplatin, edatrexate, gemcitabine, vinorelbine
etc. Those skilled in the art will recognize that other drug compounds and therapies can be
similarly be readily combined with the nucleic acid molecules of the instant invention (e.g.
ribozymes and antisense molecules) are hence within the scope of the instant invention.

20 Diagnostic uses

The nucleic acid molecules of this invention (e.g., enzymatic nucleic acid molecules)
are used as diagnostic tools to examine genetic drift and mutations within diseased cells or to
detect the presence of HIV RNA in a cell. The close relationship between enzymatic nucleic
acid molecule activity and the structure of the target RNA allows the detection of mutations
25 in any region of the molecule which alters the base-pairing and three-dimensional structure of
the target RNA. Using multiple enzymatic nucleic acid molecules described in this invention,
one maps nucleotide changes which are important to RNA structure and function *in vitro*, as
well as in cells and tissues. Cleavage of target RNAs with enzymatic nucleic acid molecules
are used to inhibit gene expression and define the role (essentially) of specified gene products
30 in the progression of disease. In this manner, other genetic targets are defined as important
mediators of the disease. These experiments lead to better treatment of the disease
progression by affording the possibility of combinational therapies (e.g., multiple enzymatic
nucleic acid molecules targeted to different genes, enzymatic nucleic acid molecules coupled
with known small molecule inhibitors, or intermittent treatment with combinations of

enzymatic nucleic acid molecules and/or other chemical or biological molecules). Other *in vitro* uses of enzymatic nucleic acid molecules of this invention are well known in the art, and include detection of the presence of mRNAs associated with HIV-related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with an enzymatic nucleic acid molecule using standard methodology.

In a specific example, enzymatic nucleic acid molecules which cleave only wild-type or mutant forms of the target RNA are used for the assay. The first enzymatic nucleic acid molecule is used to identify wild-type RNA present in the sample and the second enzymatic nucleic acid molecule is used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both enzymatic nucleic acid molecules to demonstrate the relative enzymatic nucleic acid molecule efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus each analysis requires two enzymatic nucleic acid molecules, two substrates and one unknown sample which is combined into six reactions. The presence of cleavage products is determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (*i.e.*, HIV) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the initial diagnosis. Higher mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared qualitatively or quantitatively. The use of enzymatic nucleic acid molecules in diagnostic applications contemplated by the instant invention is more fully described in George *et al.*, US Patent Nos. 5,834,186 and 5,741,679, Shih *et al.*, US Patent No. 5,589,332, Nathan *et al.*, US Patent No 5,871,914, Nathan and Ellington, International PCT publication No. WO 00/24931, Breaker *et al.*, International PCT Publication Nos. WO 00/26226 and 98/27104, and Sullenger *et al.*, International PCT publication No. WO 99/29842.

Example 10: Identification of Potential Target Sites in Human HER2 RNA

The sequence of human HER2 genes were screened for accessible sites using a computer-folding algorithm. Regions of the RNA that do not form secondary folding structures and contained potential enzymatic nucleic acid molecule and/or antisense

binding/cleavage sites were identified. The sequences of these binding/cleavage sites are shown in Tables IV and V.

Example 10: Selection of Enzymatic Nucleic Acid Cleavage Sites in Human HER2 RNA

Enzymatic nucleic acid molecule target sites were chosen by analyzing sequences of Human HER2 (Genbank accession No: X03363) and prioritizing the sites on the basis of folding. Enzymatic nucleic acid molecules were designed that can bind each target and are individually analyzed by computer folding (Christoffersen *et al.*, 1994 *J. Mol. Struct. Theochem*, 311, 273; Jaeger *et al.*, 1989, *Proc. Natl. Acad. Sci. USA*, 86, 7706) to assess whether the enzymatic nucleic acid molecule sequences fold into the appropriate secondary structure. Those enzymatic nucleic acid molecules with unfavorable intramolecular interactions between the binding arms and the catalytic core were eliminated from consideration. As noted below, variable binding arm lengths are chosen to optimize activity. Generally, at least 5 bases on each arm are able to bind to, or otherwise interact with, the target RNA.

Example 12: Chemical Synthesis and Purification of Ribozymes and Antisense for Efficient Cleavage and/or Blocking of HER2 Expression

DNAzyme molecules are designed to anneal to various sites in the RNA message. The binding arms of the DNAzyme molecules are complementary to the target site sequences described above. The DNAzymes were chemically synthesized. The method of synthesis used followed the procedure for nucleic acid synthesis as described above and in Usman *et al.*, (1987 *J. Am. Chem. Soc.*, 109, 7845), Scaringe *et al.*, (1990 *Nucleic Acids Res.*, 18, 5433) and Wincott *et al.*, *supra*, and made use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. The average stepwise coupling yields were typically >98%. The sequences of the chemically synthesized DNAzyme molecules used in this study are shown below in Table V.

Example 13: DNAzyme Cleavage of HER2 RNA Target *in vitro*

DNAzymes targeted to the human HER2 RNA are designed and synthesized as described above. These enzymatic nucleic acid molecules can be tested for cleavage activity *in vitro*, for example, using the following procedure. The target sequences and the nucleotide location within the HER2 RNA are given in Tables IV and V.

Cleavage Reactions:

Ribozymes and substrates were synthesized in 96-well format using 0.2 μ mol scale. Substrates were 5'-³²P labeled and gel purified using 7.5% polyacrylamide gels, and eluting into water. Assays were done by combining trace substrate with 500nM Ribozyme or greater, and initiated by adding final concentrations of 40mM Mg⁺², and 50mM Tris-Cl pH 8.0. For each ribozyme/substrate combination a control reaction was done to ensure cleavage was not the result of non-specific substrate degradation. A single three hour time point was taken and run on a 15% polyacrylamide gel to assess cleavage activity. Gels were dried and scanned using a Molecular Dynamics Phosphorimager and quantified using Molecular Dynamics ImageQuant software. Percent cleaved was determined by dividing values for cleaved substrate bands by full-length (uncleaved) values plus cleaved values and multiplying by 100 (%cleaved=[C/(U+C)]*100).

Example 14: DNzyme Cleavage of HER2 RNA Target *in vivo*

Cell Culture Review

The greatest HER2 specific effects have been observed in cancer cell lines that express high levels of HER2 protein (as measured by ELISA). Specifically, in one study that treated five human breast cancer cell lines with the HER2 antibody (anti-erbB2-sFv), the greatest inhibition of cell growth was seen in three cell lines (MDA-MB-361, SKBR-3 and BT-474) that express high levels of HER2 protein. No inhibition of cell growth was observed in two cell lines (MDA-MB-231 and MCF-7) that express low levels of HER2 protein (Wright, M., Grim, J., Deshane, J., Kim, M., Strong, T.V., Siegel, G.P., Curiel, D.T. (1997) An intracellular anti-erbB-2 single-chain antibody is specifically cytotoxic to human breast carcinoma cells overexpressing erbB-2. *Gene Therapy* 4: 317-322). Another group successfully used SKBR-3 cells to show HER2 antisense oligonucleotide-mediated inhibition of HER2 protein expression and HER2 RNA knockdown (Vaughn, J.P., Iglehart, J.D., Demirdji, S., Davis, P., Babiss, L.E., Caruthers, M.H., Marks, J.R. (1995) Antisense DNA downregulation of the ERBB2 oncogene measured by a flow cytometric assay. *Proc Natl Acad Sci USA* 92: 8338-8342). Other groups have also demonstrated a decrease in the levels of HER2 protein, HER2 mRNA and/or cell proliferation in cultured cells using anti-HER2 DNzymes or antisense molecules (Suzuki T., Curcio, L.D., Tsai, J. and Kashani-Sabet M. (1997) Anti-c-erb-B-2 Ribozyme for Breast Cancer. In *Methods in Molecular Medicine*, Vol. 11, Therapeutic Applications of Ribozymes, Human Press, Inc., Totowa, NJ; Weichen, K., Zimmer, C. and Dietel, M. (1997) Selection of a high activity c-erbB-2 ribozyme using a

fusion gene of c-erbB-2 and the enhanced green fluorescent protein. *Cancer Gene Therapy* 5: 45-51; Czubayko, F., Downing, S.G., Hsieh, S.S., Goldstein, D.J., Lu P.Y., Trapnell, B.C. and Wellstein, A. (1997) Adenovirus-mediated transduction of ribozymes abrogates HER-2/neu and pleiotrophin expression and inhibits tumor cell proliferation. *Gene Ther.* 4: 943-949; Colomer, R., Lupu, R., Bacus, S.S. and Gelmann, E.P. (1994) *erbB-2* antisense oligonucleotides inhibit the proliferation of breast carcinoma cells with *erbB-2* oncogene amplification. *British J. Cancer* 70: 819-825; Betram *et al.*, 1994). Because cell lines that express higher levels of HER2 have been more sensitive to anti-HER2 agents, we prefer using several medium to high expressing cell lines, including SKBR-3 and T47D, for DNAzyme screens in cell culture.

A variety of endpoints have been used in cell culture models to look at HER2-mediated effects after treatment with anti-HER2 agents. Phenotypic endpoints include inhibition of cell proliferation, apoptosis assays and reduction of HER2 protein expression. Because overexpression of HER2 is directly associated with increased proliferation of breast and ovarian tumor cells, a proliferation endpoint for cell culture assays will preferably be used as the primary screen. There are several methods by which this endpoint can be measured. Following treatment of cells with DNAzymes, cells are allowed to grow (typically 5 days) after which either the cell viability, the incorporation of [³H] thymidine into cellular DNA and/or the cell density can be measured. The assay of cell density is very straightforward and can be done in a 96-well format using commercially available fluorescent nucleic acid stains (such as Syto® 13 or CyQuant®). The assay using CyQuant® is described herein and is currently being employed to screen ~100 DNAzymes targeting HER2 (details below).

As a secondary, confirmatory endpoint a DNAzyme-mediated decrease in the level of HER2 protein expression can be evaluated using a HER2-specific ELISA.

25 *Validation of Cell Lines and DNAzyme Treatment Conditions*

Two human breast cancer cell lines (T47D and SKBR-3) that are known to express medium to high levels of HER2 protein, respectively, are considered for DNAzyme screening. In order to validate these cell lines for HER2-mediated sensitivity, both cell lines are treated with the HER2 specific antibody, Herceptin® (Genentech) and its effect on cell proliferation is determined. Herceptin® is added to cells at concentrations ranging from 0–8 µM in medium containing either no serum (OptiMem), 0.1% or 0.5% FBS and efficacy is determined *via* cell proliferation. Maximal inhibition of proliferation (~50%) in both cell lines is typically observed after addition of Herceptin® at 0.5 nM in medium containing 0.1%

or no FBS. The fact that both cell lines are sensitive to an anti-HER2 agent (Herceptin®) supports their use in experiments testing anti-HER2 DNazymes.

Prior to DNzyme screening, the choice of the optimal lipid(s) and conditions for DNzyme delivery is determined empirically for each cell line. Applicant has established a panel of cationic lipids (lipids as described in PCT application WO99/05094) that can be used to deliver DNazymes to cultured cells and are very useful for cell proliferation assays that are typically 3-5 days in length. (Additional description of useful lipids is provided above, and those skilled in the art are also familiar with a variety of lipids that can be used for delivery of oligonucleotide to cells in culture.) Initially, this panel of lipid delivery vehicles is screened in SKBR-3 and T47D cells using previously established control oligonucleotides. Specific lipids and conditions for optimal delivery are selected for each cell line based on these screens. These conditions are used to deliver HER2 specific DNazymes to cells for primary (inhibition of cell proliferation) and secondary (decrease in HER2 protein) efficacy endpoints.

Primary Screen: Inhibition of Cell Proliferation

DNzyme screens are performed using an automated, high throughput 96-well cell proliferation assay. Cell proliferation is measured over a 5-day treatment period using the nucleic acid stain CyQuant® for determining cell density. The growth of cells treated with DNzyme/lipid complexes is compared to both untreated cells and to cells treated with Scrambled-arm Attenuated core Controls. SACs can no longer bind to the target site due to the scrambled arm sequence and have nucleotide changes in the core that greatly diminish DNzyme cleavage. These SACs are used to determine non-specific inhibition of cell growth caused by DNzyme chemistry (*i.e.* multiple 2' O-Me modified nucleotides and a 3' inverted abasic). Lead DNazymes are chosen from the primary screen based on their ability to inhibit cell proliferation in a specific manner. Dose response assays are carried out on these leads and a subset was advanced into a secondary screen using the level of HER2 protein as an endpoint.

Secondary Screen: Decrease in HER2 Protein and/or RNA

A secondary screen that measures the effect of anti-HER2 DNazymes on HER2 protein and/or RNA levels is used to affirm preliminary findings. A robust HER2 ELISA for both T47D and SKBR-3 cells has been established and is available for use as an additional endpoint. In addition, a real time RT-PCR assay (TaqMan assay) has been developed to assess HER2 RNA reduction compared to an actin RNA control. Dose response activity of

nucleic acid molecules of the instant invention can be used to assess both HER2 protein and RNA reduction endpoints.

DNAzyme Mechanism Assays

5 A TaqMan® assay for measuring the DNAzyme-mediated decrease in HER2 RNA has also been established. This assay is based on PCR technology and can measure in real time the production of HER2 mRNA relative to a standard cellular mRNA such as GAPDH. This RNA assay is used to establish proof that lead DNAzymes are working through an RNA cleavage mechanism and result in a decrease in the level of HER2 mRNA, thus leading to a decrease in cell surface HER2 protein receptors and a subsequent decrease in tumor cell
10 proliferation.

Animal Models

Evaluating the efficacy of anti-HER2 agents in animal models is an important prerequisite to human clinical trials. As in cell culture models, the most HER2 sensitive mouse tumor xenografts are those derived from human breast carcinoma cells that express
15 high levels of HER2 protein. In a recent study, nude mice bearing BT-474 xenografts were sensitive to the anti-HER2 humanized monoclonal antibody Herceptin®, resulting in an 80% inhibition of tumor growth at a 1 mg kg dose (ip, 2 X week for 4-5 weeks). Tumor eradication was observed in 3 of 8 mice treated in this manner (Baselga, J., Norton, L. Albanell, J., Kim, Y.M. and Mendelsohn, J. (1998) Recombinant humanized anti-HER2
20 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res.* 15: 2825-2831). This same study compared the efficacy of Herceptin® alone or in combination with the commonly used chemotherapeutics, paclitaxel or doxorubicin. Although, all three anti-HER2 agents caused modest inhibition of tumor growth, the greatest antitumor activity was
25 produced by the combination of Herceptin® and paclitaxel (93% inhibition of tumor growth vs 35% with paclitaxel alone). The above studies provide proof that inhibition of HER2 expression by anti-HER2 agents causes inhibition of tumor growth in animals. Lead anti-HER2 DNAzymes chosen from *in vitro* assays are further tested in mouse xenograft models. DNAzymes are first tested alone and then in combination with standard chemotherapies.

30 *Animal Model Development*

Three human breast tumor cell lines (T47D, SKBR-3 and BT-474) were characterized to establish their growth curves in mice. These three cell lines have been implanted into the

mammary papillae of both nude and SCID mice and primary tumor volumes are measured 3 times per week. Growth characteristics of these tumor lines using a Matrigel implantation format can also be established. The use of two other breast cell lines that have been engineered to express high levels of HER2 can also be used in the described studies. The tumor cell line(s) and implantation method that supports the most consistent and reliable tumor growth is used in animal studies testing the lead HER2 DNAzyme(s). DNAzymes are administered by daily subcutaneous injection or by continuous subcutaneous infusion from Alzet mini osmotic pumps beginning 3 days after tumor implantation and continuing for the duration of the study. Group sizes of at least 10 animals are employed. Efficacy is determined by statistical comparison of tumor volume of DNAzyme-treated animals to a control group of animals treated with saline alone. Because the growth of these tumors is generally slow (45-60 days), an initial endpoint is the time in days it takes to establish an easily measurable primary tumor (i.e. 50-100 mm³) in the presence or absence of DNAzyme treatment.

15 Clinical Summary

Overview

Breast cancer is a common cancer in women and also occurs in men to a lesser degree. The incidence of breast cancer in the United States is ~180,000 cases per year and ~46,000 die each year of the disease. In addition, 21,000 new cases of ovarian cancer per year lead to ~13,000 deaths (data from Hung, M.-C., Matin, A., Zhang, Y., Xing, X., Sorgi, F., Huang, L. and Yu, D. (1995) HER-2/neu-targeting gene therapy - a review. *Gene* 159: 65-71 and the Surveillance, Epidemiology and End Results Program, NCI Surveillance, Epidemiology and End Results Program (SEER) Cancer Statistics Review: http://www.seer.ims.nci.nih.gov/Publications/CSR1973_1996/). Ovarian cancer is a potential secondary indication for anti-HER2 DNAzyme therapy.

A full review of breast cancer is given in the NCI PDQ for Breast Cancer (NCI PDQ/Treatment/Health Professionals/Breast Cancer: http://cancernet.nci.nih.gov/clinpdq/soa/Breast_cancer_Physician.html; NCI PDQ/Treatment/Patients/Breast Cancer: http://cancernet.nci.nih.gov/clinpdq/pif/Breast_cancer_Patient.html). A brief overview is given here. Breast cancer is evaluated or "staged" on the basis of tumor size, and whether it has spread to lymph nodes and/or other parts of the body. In Stage I breast cancer, the cancer

is no larger than 2 centimeters and has not spread outside of the breast. In Stage II, the patient's tumor is 2-5 centimeters but cancer may have spread to the axillary lymph nodes. By Stage III, metastasis to the lymph nodes is typical, and tumors are ≥ 5 centimeters. Additional tissue involvement (skin, chest wall, ribs, muscles *etc.*) may also be noted. Once
5 cancer has spread to additional organs of the body, it is classed as Stage IV.

Almost all breast cancers (>90%) are detected at Stage I or II, but 31% of these are already lymph node positive. The 5-year survival rate for node negative patients (with standard surgery/radiation/chemotherapy /hormone regimens) is 97%; however, involvement of the lymph nodes reduces the 5-year survival to only 77%. Involvement of other organs
10 (\geq Stage III) drastically reduces the overall survival, to 22% at 5 years. Thus, chance of recovery from breast cancer is highly dependent on early detection. Because up to 10% of breast cancers are hereditary, those with a family history are considered to be at high risk for breast cancer and should be monitored very closely.

Therapy

15 Breast cancer is highly treatable and often curable when detected in the early stages. (For a complete review of breast cancer treatments, see the NCI PDQ for Breast Cancer.) Common therapies include surgery, radiation therapy, chemotherapy and hormonal therapy. Depending upon many factors, including the tumor size, lymph node involvement and location of the lesion, surgical removal varies from lumpectomy (removal of the tumor and
20 some surrounding tissue) to mastectomy (removal of the breast, lymph nodes and some or all of the underlying chest muscle). Even with successful surgical resection, as many as 21% of the patients may ultimately relapse (10-20 years). Thus, once local disease is controlled by surgery, adjuvant radiation treatments, chemotherapies and/or hormonal therapies are typically used to reduce the rate of recurrence and improve survival. The therapy regimen
25 employed depends not only on the stage of the cancer at its time of removal, but other variables such the type of cancer (ductal or lobular), whether lymph nodes were involved and removed, age and general health of the patient and if other organs are involved.

Common chemotherapies include various combinations of cytotoxic drugs to kill the cancer cells. These drugs include paclitaxel (Taxol), docetaxel, cisplatin, methotrexate,
30 cyclophosphamide, doxorubin, fluorouracil *etc.* Significant toxicities are associated with these cytotoxic therapies. Well-characterized toxicities include nausea and vomiting,

myelosuppression, alopecia and mucosity. Serious cardiac problems are also associated with certain of the combinations, *e.g.* doxorubin and paclitaxel, but are less common.

Testing for estrogen and progesterone receptors helps to determine whether certain anti-hormone therapies might be helpful in inhibiting tumor growth. If either or both receptors are present, therapies to interfere with the action of the hormone ligands, can be given in combination with chemotherapy and are generally continued for several years. These adjuvant therapies are called SERMs, selective estrogen receptor modulators, and they can give beneficial estrogen-like effects on bone and lipid metabolism while antagonizing estrogen in reproductive tissues. Tamoxifen is one such compound. The primary toxic effect associated with the use of tamoxifen is a 2 to 7-fold increase in the rate of endometrial cancer. Blood clots in the legs and lung and the possibility of stroke are additional side effects. However, tamoxifen has been determined to reduce breast cancer incidence by 49% in high-risk patients and an extensive, somewhat controversial, clinical study is underway to expand the prophylactic use of tamoxifen. Another SERM, raloxifene, was also shown to reduce the incidence of breast cancer in a large clinical trial where it was being used to treat osteoporosis. In additional studies, removal of the ovaries and/or drugs to keep the ovaries from working are being tested.

Bone marrow transplantation is being studied in clinical trials for breast cancers that have become resistant to traditional chemotherapies or where >3 lymph nodes are involved. Marrow is removed from the patient prior to high-dose chemotherapy to protect it from being destroyed, and then replaced after the chemotherapy. Another type of "transplant" involves the exogenous treatment of peripheral blood stem cells with drugs to kill cancer cells prior to replacing the treated cells in the bloodstream.

One biological treatment, a humanized monoclonal anti-HER2 antibody, Herceptin® (Genentech) has been approved by the FDA as an additional treatment for HER2 positive tumors. Herceptin® binds with high affinity to the extracellular domain of HER2 and thus blocks its signaling action. Herceptin® can be used alone or in combination with chemotherapeutics (*i.e.* paclitaxel, docetaxel, cisplatin, *etc.*) (Pegram, M.D., Lipton, A., Hayes, D.F., Weber, B.L., Baselga, J.M., Tripathy, D., Baly, D., Baughman, S.A., Twaddell, T., Glaspy, J.A. and Slamon, D.J. (1998) Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *J. Clin. Oncol.* 16: 2659-2671). In Phase III studies, Herceptin® significantly improved the response rate to chemotherapy as well as improving the time to

progression (Ross, J.S. and Fletcher, J.A. (1998) The HER-2/neu oncogene in breast cancer: Prognostic factor, predictive factor and target for therapy. *Oncologist* 3: 1998). The most common side effects attributed to Herceptin® are fever and chills, pain, asthenia, nausea, vomiting, increased cough, diarrhea, headache, dyspnea, infection, rhinitis, and insomnia.

5 Herceptin® in combination with chemotherapy (paclitaxel) can lead to cardiotoxicity (Sparano, J.A. (1999) Doxorubicin/taxane combinations: Cardiac toxicity and pharmacokinetics. *Semin. Oncol.* 26: 14-19), leukopenia, anemia, diarrhea, abdominal pain and infection.

HER2 Protein Levels for Patient Screening and as a Potential Endpoint

10 Because elevated HER2 levels can be detected in at least 30% of breast cancers, breast cancer patients can be pre-screened for elevated HER2 prior to admission to initial clinical trials testing an anti-HER2 DNAzyme. Initial HER2 levels can be determined (by ELISA) from tumor biopsies or resected tumor samples.

During clinical trials, it may be possible to monitor circulating HER2 protein by ELISA

15 (Ross and Fletcher, 1998). Evaluation of serial blood/serum samples over the course of the anti-HER2 DNAzyme treatment period could be useful in determining early indications of efficacy. In fact, the clinical course of Stage IV breast cancer was correlated with shed HER2 protein fragment following a dose-intensified paclitaxel monotherapy. In all responders, the HER2 serum level decreased below the detection limit (Luftner, D., Schnabel, S. and

20 Possinger, K. (1999) c-erbB-2 in serum of patients receiving fractionated paclitaxel chemotherapy. *Int. J. Biol. Markers* 14: 55-59).

Two cancer-associated antigens, CA27.29 and CA15.3, can also be measured in the serum. Both of these glycoproteins have been used as diagnostic markers for breast cancer. CA27.29 levels are higher than CA15.3 in breast cancer patients; the reverse is true in healthy

25 individuals. Of these two markers, CA27.29 was found to better discriminate primary cancer from healthy subjects. In addition, a statistically significant and direct relationship was shown between CA27.29 and large vs small tumors and node positive vs node negative disease (Gion, M., Mione, R., Leon, A.E. and Dittadi, R. (1999) Comparison of the diagnostic accuracy of CA27.29 and CA15.3 in primary breast cancer. *Clin. Chem.* 45: 630-637).

30 Moreover, both cancer antigens were found to be suitable for the detection of possible metastases during follow-up (Rodriguez de Paterna, L., Arnaiz, F., Estenoz, J. Ortuno, B. and Lanzos E. (1999) Study of serum tumor markers CEA, CA15.3, CA27.29 as diagnostic parameters in patients with breast carcinoma. *Int. J. Biol. Markers* 10: 24-29). Thus,

- blocking breast tumor growth may be reflected in lower CA27.29 and/or CA15.3 levels compared to a control group. FDA submissions for the use of CA27.29 and CA15.3 for monitoring metastatic breast cancer patients have been filed (reviewed in Beveridge, R.A. (1999) Review of clinical studies of CA27.29 in breast cancer management. *Int. J. Biol. Markers* 14: 36-39). Fully automated methods for measurement of either of these markers are commercially available.

Indications

- Particular degenerative and disease states that can be associated with HER2 expression modulation include but are not limited to cancer, for example breast cancer and ovarian cancer and/or any other diseases or conditions that are related to or will respond to the levels of HER2 in a cell or tissue, alone or in combination with other therapies

The present body of knowledge in HER2 research indicates the need for methods to assay HER2 activity and for compounds that can regulate HER2 expression for research, diagnostic, and therapeutic use.

- The use of monoclonal antibodies, chemotherapy, radiation therapy, and analgesics, are all non-limiting examples of methods that can be combined with or used in conjunction with the nucleic acid molecules (e.g. DNazymes) of the instant invention. Common chemotherapies that can be combined with nucleic acid molecules of the instant invention include various combinations of cytotoxic drugs to kill cancer cells. These drugs include but are not limited to paclitaxel (Taxol), docetaxel, cisplatin, methotrexate, cyclophosphamide, doxorubin, fluorouracil carboplatin, edatrexate, gemcitabine, vinorelbine etc. Those skilled in the art will recognize that other drug compounds and therapies can be similarly be readily combined with the nucleic acid molecules of the instant invention (e.g. DNzyme molecules) are hence within the scope of the instant invention.

Diagnostic uses

- The nucleic acid molecules of this invention (e.g., enzymatic nucleic acid molecules) can be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of HER2 RNA in a cell. The close relationship between enzymatic nucleic acid molecule activity and the structure of the target RNA allows the detection of mutations in any region of the molecule that alters the base-pairing and three-dimensional structure of the target RNA. By using multiple enzymatic nucleic acid molecules described in this invention, one can map nucleotide changes which are important to RNA structure and

function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with enzymatic nucleic acid molecules can be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets can be defined as important mediators of the disease. These experiments can lead to better treatment of the disease progression by affording the possibility of combinational therapies (e.g., multiple enzymatic nucleic acid molecules targeted to different genes, enzymatic nucleic acid molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations of enzymatic nucleic acid molecules and/or other chemical or biological molecules). Other *in vitro* uses of enzymatic nucleic acid molecules of this invention are well known in the art, and include detection of the presence of mRNAs associated with HER2-related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with an enzymatic nucleic acid molecule using standard methodology.

In a specific example, enzymatic nucleic acid molecules that cleave only wild-type or mutant forms of the target RNA are used for the assay. The first enzymatic nucleic acid molecule is used to identify wild-type RNA present in the sample and the second enzymatic nucleic acid molecule is used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both enzymatic nucleic acid molecules to demonstrate the relative enzymatic nucleic acid molecule efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus each analysis requires two enzymatic nucleic acid molecules, two substrates and one unknown sample which is combined into six reactions. The presence of cleavage products is determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (*i.e.*, HER2) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the initial diagnosis. Higher mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared qualitatively or quantitatively. The use of enzymatic nucleic acid molecules in diagnostic applications contemplated by the instant invention is more fully described in George *et al.*, US Patent Nos. 5,834,186 and 5,741,679, Shih *et al.*, US Patent No. 5,589,332, Nathan *et al.*, US Patent No 5,871,914, Nathan and Ellington, International PCT publication

No. WO 00/24931, Breaker *et al.*, International PCT Publication Nos. WO 00/26226 and 98/27104, and Sullenger *et al.*, International PCT publication No. WO 99/29842.

Additional Uses

5 Potential uses of sequence-specific enzymatic nucleic acid molecules of the instant invention can have many of the same applications for the study of RNA that DNA restriction endonucleases have for the study of DNA (Nathans *et al.*, 1975 *Ann. Rev. Biochem.* 44:273). For example, the pattern of restriction fragments can be used to establish sequence relationships between two related RNAs, and large RNAs can be specifically cleaved to
10 fragments of a size more useful for study. The ability to engineer sequence specificity of the enzymatic nucleic acid molecule is ideal for cleavage of RNAs of unknown sequence. Applicant has described the use of nucleic acid molecules to modulate gene expression of target genes in bacterial, microbial, fungal, viral, and eukaryotic systems including plant or mammalian cells.

15 All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

20 One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

25 It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims.

30 The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting

essentially of" and "consisting of" can be replaced with either of the other two terms. The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized
5 that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed can be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description
10 and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

15 Other embodiments are within the claims that follow.

Table I:

A. 2.5 μ mol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	6.5	163 μ L	45 sec	2.5 min	7.5 min
S-Ethyl Tetrazole	23.8	238 μ L	45 sec	2.5 min	7.5 min
Acetic Anhydride	100	233 μ L	5 sec	5 sec	5 sec
N-Methyl Imidazole	186	233 μ L	5 sec	5 sec	5 sec
TCA	176	2.3 mL	21 sec	21 sec	21 sec
Iodine	11.2	1.7 mL	45 sec	45 sec	45 sec
Beaucage	12.9	645 μ L	100 sec	300 sec	300 sec
Acetonitrile	NA	6.67 mL	NA	NA	NA

B. 0.2 μ mol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	15	31 μ L	45 sec	233 sec	465 sec
S-Ethyl Tetrazole	38.7	31 μ L	45 sec	233 min	465 sec
Acetic Anhydride	655	124 μ L	5 sec	5 sec	5 sec
N-Methyl Imidazole	1245	124 μ L	5 sec	5 sec	5 sec
TCA	700	732 μ L	10 sec	10 sec	10 sec
Iodine	20.6	244 μ L	15 sec	15 sec	15 sec
Beaucage	7.7	232 μ L	100 sec	300 sec	300 sec
Acetonitrile	NA	2.64 mL	NA	NA	NA

C. 0.2 μ mol Synthesis Cycle 96 well Instrument

Reagent	Equivalents:DNA/ 2'-O-methyl/Ribo	Amount: DNA/2'-O- methyl/Ribo	Wait Time* DNA	Wait Time* 2'-O- methyl	Wait Time* Ribo
Phosphoramidites	22/33/66	40/60/120 μ L	60 sec	180 sec	360sec
S-Ethyl Tetrazole	70/105/210	40/60/120 μ L	60 sec	180 min	360 sec
Acetic Anhydride	265/265/265	50/50/50 μ L	10 sec	10 sec	10 sec
N-Methyl Imidazole	502/502/502	50/50/50 μ L	10 sec	10 sec	10 sec
TCA	238/475/475	250/500/500 μ L	15 sec	15 sec	15 sec
Iodine	6.8/6.8/6.8	80/80/80 μ L	30 sec	30 sec	30 sec
Beaucage	34/51/51	80/120/120	100 sec	200 sec	200 sec
Acetonitrile	NA	1150/1150/1150 μ L	NA	NA	NA

- Wait time does not include contact time during delivery.

Table V: Human HER2 Synthetic DNzyme and Target molecules

Gene	Pos	Target	Seq ID	RPI#	DNzyme	Seq ID
erbB2	377	CCACCA A UGCCAG	6632	24998	cuggca GGCTAGCTACAACGA uggugg B	6637
erbB2	766	UUCUCCG A UGUGUAA	6633	24999	uuacaca GGCTAGCTACAACGA cggagaa B	6638
erbB2	1202	UGUGCU A UGGUCU	6634	25000	agacca GGCTAGCTACAACGA agcaca B	6639
erbB2	1444	CCUCAGC G UCUUCCA	6635	25001	uggaaga GGCTAGCTACAACGA gcugagg B	6640
erbB2	1583	AUCCACC A UAACACC	6636	25002	gguguua GGCTAGCTACAACGA gguggau B	6641

A, G, C, T (*italic*) = deoxy

lower case = 2'-O-methyl

B = inverted deoxybasic derivative

Table VI: Human HIV Hammerhead Ribozyme and Substrate Sequence

Substrate	Seq ID	Hammerhead	Seq ID
AUAAAGCU U GCCUUGAG	6642	CUCAAGGC CUGAUGAGGCCG <u>UUAGGCCGAA</u> AGCUUUUAU	6727
AGGCUAAU U UUUUAGGG	6643	CCCUAAAA CUGAUGAGGCCG <u>UUAGGCCGAA</u> AUUAGCCU	6728
GGCUAAU U UUUAGGGA	6644	UCCCUAAA CUGAUGAGGCCG <u>UUAGGCCGAA</u> AAUUAGCC	6729
GCCUCAAU A AAGCUUGC	6645	GCAAGCUU CUGAUGAGGCCG <u>UUAGGCCGAA</u> AUUGAGGC	6730
UUUCGGGU U UAUUACAG	6646	CUGUAAUA CUGAUGAGGCCG <u>UUAGGCCGAA</u> ACCCGAAA	6731
GCAGGACU C GGCUUGC	6647	AGCAAGCC CUGAUGAGGCCG <u>UUAGGCCGAA</u> AGUCCUGC	6732

Input Sequence = HIV1. Cut Site = UH/.

Arm Length = 8. Core Sequence = CUGAUGAG GCCGUUAGGC CGAA

HIV1 Consensus

Underlined region can be any X sequence or linker, as described herein.

Table VII: Human HIV Inozyme and Substrate Sequence

Substrate	Seq ID	Inozyme	Seq ID
UGGAAAAC A GAUGGCAG	6648	CUGCCAUC CUGAUGAGGCCGUAAGGCCGAA IUUUUCCA	6733
AAUAAAGC U UGCCUUGA	6649	UCAAGGCA CUGAUGAGGCCGUAAGGCCGAA ICUUUAUU	6734
UCUCUAGC A GUGGCGCC	6650	GGCGCCAC CUGAUGAGGCCGUAAGGCCGAA ICUAGAGA	6735
GGAGCCAC C CCACAAGA	6651	UCUUGUGG CUGAUGAGGCCGUAAGGCCGAA IUGGCUCC	6736
AGUGGCGC C CGAACAGG	6652	CCUGUUCG CUGAUGAGGCCGUAAGGCCGAA ICGCCACU	6737
GUGGCGCC C GAACAGGG	6653	CCCUGUUC CUGAUGAGGCCGUAAGGCCGAA ICGCCAC	6738
CUCGACGC A GGACUCGG	6654	CCGAGUCC CUGAUGAGGCCGUAAGGCCGAA ICGUCGAG	6739
CGCAGGAC U CGGCUUGC	6655	GCAAGCCG CUGAUGAGGCCGUAAGGCCGAA IUCCUGCG	6740

Input Sequence = HIV1. Cut Site = CH/.

Arm Length = 8. Core Sequence = CUGAUGAG GCCGUAAGGC CGAA

HIV1 Consensus

Underlined region can be any X sequence or linker, as described herein.

"I" stands for Inosine.

Table VIII: Human HIV Zinzyme and Substrate Sequence

Substrate	Seq ID	Zinzyme	Seq ID
UCAAUAAA G CUUGCCU	6656	AAGGCAAG GCCGAAAGGCGAGUGAGGUCU UUUAUUGA	6741
AGGACUCG G CUUGCUGA	6657	UCAGCAAG GCCGAAAGGCGAGUGAGGUCU CGAGUCCU	6742
GCAGUGGC G CCCGAACA	6658	UGUUCGGG GCCGAAAGGCGAGUGAGGUCU GCCACUGC	6743
CUCUAGCA G UGCGGCC	6659	GGGCGCCA GCCGAAAGGCGAGUGAGGUCU UGCUAGAG	6744
UAGCAGUG G CGCCGAA	6660	UUCGGGCG GCCGAAAGGCGAGUGAGGUCU CACUGCUA	6745
AGAGAUGG G UGCGAGAG	6661	CUCUCGCA GCCGAAAGGCGAGUGAGGUCU CCAUCUCU	6746
AGAUGGGU G CGAGAGCG	6662	CGCUCUCG GCCGAAAGGCGAGUGAGGUCU ACCCAUCU	6747
CUCUCGAC G CAGGACUC	6663	GAGUCCUG GCCGAAAGGCGAGUGAGGUCU GUCGAGAG	6748

Input Sequence = HIV1. Cut Site = G/Y

Arm Length = 8. Core Sequence = GCcgaagGCGaGuCaagGgCu

HIV1 Consensus

Table IX: Human HIV DNAzyme and Substrate Sequence

Substrate	Seq ID	DNAzyme	Seq ID
UCAAUAAA G CUUGCCUU	6656	AAGGCAAG GGCTAGCTACAACGA TTTATTGA	6749
AGGACUCG G CUUGCUGA	6657	TCAGCAAG GGCTAGCTACAACGA CGAGTCCT	6750
GCAGUGGC G CCCGAACA	6658	TGTTGGGG GGCTAGCTACAACGA GCCACTGC	6751
CUCUAGCA G UGGCGCCC	6659	GGGCGCCA GGCTAGCTACAACGA TGCTAGAG	6752
UAGCAGUG G CGCCCGAA	6660	TTCGGGCG GGCTAGCTACAACGA CACTGCTA	6753
AGAGAUGG G UGCGAGAG	6661	CTCTCGCA GGCTAGCTACAACGA CCATCTCT	6754
AGAUGGGU G CGAGAGCG	6662	CGCTCTCG GGCTAGCTACAACGA ACCCATCT	6755
CUCUCGAC G CAGGACUC	6663	GAGTCCTG GGCTAGCTACAACGA GTCGAGAG	6756
UAUGGAAA A CAGAUGGC	6664	GCCATCTG GGCTAGCTACAACGA TTTCCATA	6757
GAAAACAG A UGGCAGGU	6665	ACCTGCCA GGCTAGCTACAACGA CTGTTTTC	6758
AAGCCUCA A UAAAGCUU	6666	AAGCTTTA GGCTAGCTACAACGA TGAGGCTT	6759
GGAGAGAG A UGGGUGCG	6667	CGCACCCA GGCTAGCTACAACGA CTCTCTCC	6760
GACGCAGG A CUCGGCUU	6668	AAGCCGAG GGCTAGCTACAACGA CCTGCGTC	6761

Input Sequence = HIV1. Cut Site = R/Y

Arm Length = 8. Core Sequence = GGCTAGCTACAACGA

HIV1 Consensus

Table X: Human HIV Amberzyme and Substrate Sequence

Substrate	Seq ID	Amberzyme	Seq ID
UCAUAAA G CUUGCCUU	6656	AAGGCAAG GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG UUUUAUGA	6762
AGGACUCG G CUUGCUGA	6657	UCAGCAAG GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CGAGUCCU	6763
GCAGUGG G CCGGAACA	6658	UGUUCGGG GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG GCCACUCC	6764
CUCUAGCA G UGGCGCCC	6659	GGCGGCTA GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG UGCUGAGG	6765
UAGCAGUG G CGCCCGAA	6660	UUCGGGCG GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CACUGCUA	6766
AGAGAUGG G UCGGAGAG	6661	CUCUGGCA GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CCAUCUCU	6767
AGAUGGUU G CGAGAGCG	6662	CGCUCUCG GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG ACCCAUCU	6768
CUCUCGAC G CAGGACUC	6663	GAGUCCUG GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG GUCGAGAG	6769
GGAAACA G AUGGCAGG	6669	CCUGCCAU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG UGUUUUCC	6770
AUGGGUG G AGAGGUC	6670	GACGUCU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG GCACCAU	6771
AAAGGGG G GAUUGGG	6671	CCCCAUC GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CCCCUCUU	6772
AGAAAGG G GGAUUGG	6672	CCAUUCC GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CCUUUUCU	6773
GAAAGGG G GGAUUGG	6673	CCCAUCC GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CCCCUCUU	6774
GGCUAGAA G GAGAGAGA	6674	UCUCUCUC GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG UUCUAGCC	6775
UUUAAAA G AAAGGGG	6675	CCCCUUU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG UUUUAAA	6776
UAUGGCAG G AAGAGCG	6676	CGUUCUU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CUGCCAUA	6777
UGCGGCC G AACAGGGA	6677	UCCUGUU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG GGGGCCCA	6778
GAGAGAUG G GUGCGAGA	6678	UUCGCGAC GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CAUCUCUC	6779
CGACGCAG G ACUCGGCU	6679	AGCCGAGU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CUGGCUUG	6780
UGACUAGC G GAGGCUAG	6680	CUAGCCUC GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG GCUAGUCA	6781
UAGAAGGA G AGAGAUGG	6681	CCAUCUCU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG UCCUUCUA	6782
AGGAGAGA G AUGGGUCC	6682	GCACCAU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG UCUUCUCU	6783
GAGGAGA G AGAUGGU	6683	ACCCAUCU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG UCUUCUUC	6784
UCGACGCA G GACUGGCG	6684	GCGAGUC GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG UGCGUCGA	6785
CUAGCAGU G GCGCCCGA	6685	UCGGGCGC GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG ACUGCUAG	6786
GACUAGCG G AGGCUAGA	6686	UUAAGCUU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CGCUAGUC	6787
GUAGAAG G AGAGAGAU	6687	AUCUCUCU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CUUCUAGC	6788
AAAGGGG G AUUGGGG	6688	CCCCCAU GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG CCCCUCUU	6789

Input Sequence = HIV1. Cut Site = G/.

Arm Length = 8. Core Sequence = GGAGGAAACUCC CU UCAAGGACAUUGUCCGGG

HIV1 Consensus

Table XI: Human HIV Enzymatic Nucleic Acid and Target molecules

Target	Seq ID	RPI#	Enzymatic Nucleic Acid	Seq ID
GAGAUGG G UGCGAGA	6718	25003	ucucgca GGCTAGCTACAACGA ccaucuc B	6790
AUGGAAA A CAGAUGG	6719	25004	ccaucug GGCTAGCTACAACGA uuuccau B	6791
AAAACAG A UGGCAGG	6720	25005	ccugcca GGCTAGCTACAACGA cuguuuu B	6792
AGCCUCA A UAAAGCU	6721	25006	agcuuua GGCTAGCTACAACGA ugaggcu B	6793
GAGAGAG A UGGGUGC	6722	25007	gcaccca GGCTAGCTACAACGA cucucuc B	6794
CAAUAAA G CUUGCCU	6723	25008	aggcaag gccgaaaggCgagugaGGuCu uuuaug B	6795
GGACUCG G CUUGCUG	6724	25009	cagcaag gccgaaaggCgagugaGGuCu cgagucc B	6796
GAGAUGG G UGCGAGA	6718	25010	ucucgca gccgaaaggCgagugaGGuCu ccaucuc B	6797
GAUGGGU G CGAGAGC	6725	25011	gcucucg gccgaaaggCgagugaGGuCu acccauc B	6798
UCUCGAC G CAGGACU	6726	25012	aguccug gccgaaaggCgagugaGGuCu gucgaga B	6799

G = Guanosine

A, G, C, T (*italic*) = deoxy

lower case = 2'-O-methyl

s = phosphorothioate 3'-internucleotide linkage

C = 2'-deoxy-2'-Amino cytidine

B = inverted deoxyabasic derivative

Table XII: Human HIV-1 Sequences

Genbank Acc#	Seq Name(s)	Subtype	Organism
A04321	IIIB LAI	B	HIV-1
AF110962	96BW0402	C	HIV-1
AF110963	96BW0407	C	HIV-1
AF110968	96BW0504	C	HIV-1
AF110965	96BW0409	C	HIV-1
AF110966	96BW0410	C	HIV-1
AF110964	96BW0408	C	HIV-1
AF110975	96BW15C05	C	HIV-1
AF110974	96BW15C02	C	HIV-1
AF110973	96BW15B03	C	HIV-1
AF107771	UGSE8131	A	HIV-1
U69585	WCIPR854	B	HIV-1
U69588	WCIPR855	B	HIV-1
U69589	WCIPR9011	B	HIV-1
U69591	WCIPR9018	B	HIV-1
U69592	WCIPR9031	B	HIV-1
U69593	WCIPR9032	B	HIV-1
U69586	WCIPR8546	B	HIV-1
AF003888	NL43WC001	B	HIV-1
X01762	REHTLV3 LAI IIIB	B	HIV-1
AF075719	MNTQ MNcloneTQ	B	HIV-1
AJ239083	97CAMP645MO	MO	HIV-1
D86069	PM213	B	HIV-1
K02083	PV22	B	HIV-1
M93259	YU10	B	HIV-1
Z11530	F12CG	B	HIV-1
AB032740	TH022 95TNIH022	CRF01_AE	HIV-1
AF107770	SE7812	CRF02_AG	HIV-1
AF070521	NL43E9	B	HIV-1
AF033819	HXB2-copy LAI	B	HIV-1
AF003887	WC001	B	HIV-1
AF069140	DH123	B	HIV-1
AF110967	96BW0502	C	HIV-1
K03455	HXB2 HXB2CG	B	HIV-1
M96155	P896 89.6	B	HIV-1
X04415	MAL MALCG	ADK	HIV-1
AF133821	MB2059	D	HIV-1
D86068	MCK1	B	HIV-1
U69587	WCIPR8552	B	HIV-1
U69580	WCIPR9012	B	HIV-1
AB032741	95TNIH047 TH047	CRF01_AE	HIV-1
AB023804	93IN101	C	HIV-1
AF193275	97BL006	A	HIV-1
AF197340	90CF11697	CRF01_AE	HIV-1
AF224507	WK	B	HIV-1
AJ271445	GB8 GB8-46R	B	HIV-1
AF197338	93TH057	CRF01_AE	HIV-1
AF197339	93TH065	CRF01_AE	HIV-1
AF197341	90CF4071	CRF01_AE	HIV-1

U69584	85WCIPR54	B	HIV-1
L31963	TH475A LAI	B	HIV-1
U46016	ETH2220 C2220	C	HIV-1
U21135	WEAU160 GHOSH	B	HIV-1
AF042106	MBCC18R01	B	HIV-1
K03454	ELI	D	HIV-1
U51188	90CF402 90CR402	CRF01_AE	HIV-1
U51189	93TH253	CRF01_AE	HIV-1
U34603	H0320-2A12	B	HIV-1
M38429	JRCSF JR-CSF	B	HIV-1
M17451	RF HAT3	B	HIV-1
L02317	BC BCSG3	B	HIV-1
M93258	YU2 YU2X	B	HIV-1
M22639	Z2Z6 Z2 CDC-Z34	D	HIV-1
AF004394	AD8, AD87 ADA	B	HIV-1
AF049337	94CY032-3	CRF04_cpx	HIV-1
U34604	3202A21	B	HIV-1
L20587	ANT70	O	HIV-1
D10112	CAM1	B	HIV-1
U54771	CM240	CRF01_AE	HIV-1
U43096	D31	B	HIV-1
U37270	C18MBC	B	HIV-1
U43141	HAN	B	HIV-1
U23487	MANC	B	HIV-1
M17449	MNCG MN	B	HIV-1
L20571	MVP5180	O	HIV-1
M27323	NDK	D	HIV-1
M38431	NY5CG	B	HIV-1
M28727	OYI, 397	B	HIV-1
K02007	SF2 LAV2 ARV2	B	HIV-1
M62320	U455 U455A	A	HIV-1
U26546	WR27	B	HIV-1
AF004885	Q23	A	HIV-1
AF042100	MBC200	B	HIV-1
AF042101	MBC925	B	HIV-1
AJ006287	89SP061 89ES061	B	HIV-1
AF067154	93IN999 301999	C	HIV-1
AF067155	95IN21068 21068	C	HIV-1
AJ006022	YBF30	N	HIV-1
AF061642	SE6165 G6165	G	HIV-1
AF119820	97PVCH GR11	CRF04_cpx	HIV-1
AF119819	97PVMY GR84	CRF04_cpx	HIV-1
K02013	LAI BRU	B	HIV-1
L39106	IBNG	CRF02_AG	HIV-1
U12055	LW123	B	HIV-1
M19921	NL43 pNL43	B	HIV-1
AF061640	HH8793-1.1	G	HIV-1
AF061641	HH8793-12.1	G	HIV-1
AF063223	DJ263	CRF02_AG	HIV-1
AF049495	NC7	B	HIV-1
AF049494	499JC16	B	HIV-1
AF086817	TWCYS LM49	B	HIV-1
AF064699	BFP90	CRF06_cpx	HIV-1

AF084936	DRCBL	G	HIV-1
AF193253	VI1310 AF193253	CRF05_DF	HIV-1
AF190127	VI991	H	HIV-1
AF193276	KAL153-2	CRF03_AB	HIV-1
AF192135	BW2117	AJ	HIV-1
AJ288982	95ML127	CRF06_cpx	HIV-1
AJ288981	97SE1078	CRF06_cpx	HIV-1
AJ271370	YBF106	N	HIV-1
AJ237565	97NOGIL3	ADHK	HIV-1

CLAIMS

What we claim is:

1. A siRNA nucleic acid molecule that modulates expression of a nucleic acid molecule encoding HER2.
2. A enzymatic nucleic acid molecule that modulates expression of a nucleic acid molecule encoding HER2.
3. An enzymatic nucleic acid molecule comprising a sequence selected from the group consisting of SEQ ID NOs: 5644-6631 and 6637-6641.
4. An enzymatic nucleic acid molecule comprising at least one binding arm wherein one or more of said binding arms comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs: 4656-5643 and 6632-6636.
5. A siRNA nucleic acid molecule comprising a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs: 4656-5643 and 6632-6636.
6. The nucleic acid molecule of any of claims 1-5, wherein said nucleic acid molecule is adapted to treat cancer.
7. The enzymatic nucleic acid molecule of any of claims 2-4, wherein said enzymatic nucleic acid molecule has an endonuclease activity to cleave RNA having HER2 sequence.
8. The enzymatic nucleic acid molecule of claim 2, wherein said enzymatic nucleic acid molecule is a DNAzyme in a 10-23 configuration.
9. The enzymatic nucleic acid molecule of claim 8, wherein said enzymatic nucleic acid molecule comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs: 4656-5643 and 6632-6636.

10. The enzymatic nucleic acid molecule of claim 8, wherein said enzymatic nucleic acid molecule comprises a sequence selected from the group consisting of SEQ ID NOs: 5644-6631 and 6637-6641.
11. The nucleic acid molecule of any of claims 1, 2, 4 or 5, wherein said nucleic acid molecule comprises between 12 and 100 bases complementary to a RNA having HER2 sequence.
12. The nucleic acid molecule of claim of any of claims 1, 2, 4 or 5, wherein said nucleic acid molecule comprises between 14 and 24 bases complementary to a RNA having HER2 sequence.
13. The nucleic acid molecule of any of claims 1-5, wherein said nucleic acid molecule is chemically synthesized.
14. The nucleic acid molecule of any of claims 1-5, wherein said nucleic acid molecule comprises at least one 2'-sugar modification.
15. The nucleic acid molecule of any of claims 1-5, wherein said nucleic acid molecule comprises at least one nucleic acid base modification.
16. The nucleic acid molecule of any of claims 1-5, wherein said nucleic acid molecule comprises at least one phosphate backbone modification.
17. A mammalian cell comprising the nucleic acid molecule of any of claims 1-5.
18. The mammalian cell of claim 17, wherein said mammalian cell is a human cell.
19. A method of reducing HER2 activity in a cell, comprising contacting said cell with the nucleic acid molecule of any of claims 1-5, under conditions suitable for said reduction of HER2 activity.
20. A method of treatment of a subject having a condition associated with the level of HER2, comprising contacting cells of said subject with the nucleic acid molecule of any of claims 1-5, under conditions suitable for said treatment.
21. The method of claim 20 further comprising the use of one or more drug therapies under conditions suitable for said treatment.

22. A method of cleaving RNA having HER2 sequence comprising contacting an enzymatic nucleic acid molecule of any of claims 2-4 with said RNA under conditions suitable for the cleavage.
23. The method of claim 22, wherein said cleavage is carried out in the presence of a divalent cation.
24. The method of claim 23, wherein said divalent cation is Mg^{2+} .
25. The nucleic acid molecule of any of claims 1-5, wherein said nucleic acid molecule comprises a cap structure, wherein the cap structure is at the 5'-end, 3'-end, or both the 5'-end and the 3'-end of said nucleic acid molecule.
26. The nucleic acid molecule of claim 25, wherein the cap structure at the 5'-end, 3'-end, or both the 5'-end and the 3'-end comprises a 3',3'-linked or 5',5'-linked deoxyabasic ribose derivative.
27. An expression vector comprising a nucleic acid sequence encoding at least one nucleic acid molecule of any of claims 1-5 in a manner that allows expression of the nucleic acid molecule.
28. A mammalian cell comprising an expression vector of claim 27.
29. The mammalian cell of claim 28, wherein said mammalian cell is a human cell.
30. The expression vector of claim 27, wherein said nucleic acid molecule is in a DNAzyme configuration.
31. The expression vector of claim 27, wherein said expression vector further comprises a sequence for a nucleic acid molecule complementary to a nucleic acid molecule having HER2 sequence.
32. The expression vector of claim 27, wherein said expression vector comprises a nucleic acid sequence encoding two or more of said nucleic acid molecules, which may be the same or different.
33. The expression vector of claim 32, wherein said expression vector further comprises a sequence encoding an antisense nucleic acid molecule or siRNA molecule complementary to a nucleic acid molecule having HER2 sequence.

34. A method for treatment of cancer comprising administering to a subject the nucleic acid molecule of any of claims 1-5 under conditions suitable for said treatment.
35. The method of claim 34, wherein said cancer is breast cancer.
36. The method of claim 34, wherein said cancer is ovarian cancer.
37. The method of claim 34, wherein said method further comprises administering to said subject one or more other therapies under conditions suitable for said treatment.
38. The method of claim 21 wherein said other drug therapies are chosen from monoclonal antibody therapy, chemotherapy, radiation therapy, and analgesic therapy.
39. The method of claim 37 wherein said other drug therapies are chosen from monoclonal antibody therapy, chemotherapy, radiation therapy, and analgesic therapy.
40. The method of claim 38, wherein said chemotherapy is selected from the group consisting of paclitaxel (Taxol), docetaxel, cisplatin, methotrexate, cyclophosphamide, doxorubin, fluorouracil carboplatin, edatrexate, gemcitabine, and vinorelbine.
41. The method of claim 38, wherein said monoclonal antibody is Herceptin (trastuzumab).
42. The method of claim 39, wherein said chemotherapy is selected from the group consisting of paclitaxel (Taxol), docetaxel, cisplatin, methotrexate, cyclophosphamide, doxorubin, fluorouracil carboplatin, edatrexate, gemcitabine, and vinorelbine.
43. The method of claim 39, wherein said monoclonal antibody is Herceptin (trastuzumab).
44. A composition comprising a nucleic acid molecule of any of claims 1-5 in a pharmaceutically acceptable carrier.

45. A method of administering to a cell a nucleic acid molecule of any of claims 1-5 comprising contacting said cell with the nucleic acid molecule under conditions suitable for said administration.
46. The method of claim 45, wherein said cell is a mammalian cell.
47. The method of claim 45, wherein said cell is a human cell.
48. The method of claim 45, wherein said administration is in the presence of a delivery reagent.
49. The method of claim 48, wherein said delivery reagent is a lipid.
50. The method of claim 49, wherein said lipid is a cationic lipid.
51. The method of claim 49, wherein said lipid is a phospholipid.
52. The method of claim 48, wherein said delivery reagent is a liposome.
53. A siRNA nucleic acid molecule that modulates expression of a nucleic acid molecule encoding K-Ras.
54. A siRNA nucleic acid molecule that modulates expression of a nucleic acid molecule encoding H-Ras or N-Ras.
55. An enzymatic nucleic acid molecule that modulates expression of a nucleic acid molecule encoding K-Ras.
56. An enzymatic nucleic acid molecule that modulates expression of a nucleic acid molecule encoding H-Ras or N-Ras.
57. An enzymatic nucleic acid molecule comprising a sequence of SEQ ID NOs: 2329-4655.
58. An enzymatic nucleic acid molecule comprising at least one binding arm wherein one or more of said binding arms comprises a sequence complementary to a sequence of SEQ ID NOs: 1-2328.
59. A siRNA nucleic acid molecule comprising a sequence complementary to a sequence of SEQ ID NOs: 1-2328.

60. The nucleic acid molecule of any of claims 53-59, wherein said nucleic acid molecule is adapted to treat cancer.
61. The enzymatic nucleic acid molecule of any of claims 55, 57 or 58, wherein said enzymatic nucleic acid molecule has an endonuclease activity to cleave RNA having a K-Ras sequence.
62. The enzymatic nucleic acid molecule of any of claims 56-58, wherein said enzymatic nucleic acid molecule has an endonuclease activity to cleave RNA having an H-Ras sequence.
63. The enzymatic nucleic acid molecule of claim 55 or claim 56, wherein said enzymatic nucleic acid molecule is a DNAzyme in a 10-23 configuration.
64. The enzymatic nucleic acid molecule of claim 63, wherein said enzymatic nucleic acid molecule comprises a sequence complementary to a sequence of SEQ ID NOs: 1-2328.
65. The enzymatic nucleic acid molecule of claim 63, wherein said enzymatic nucleic acid molecule comprises a sequence of SEQ ID NOs: 2329-4655.
66. The nucleic acid molecule of any of claims 53-59, wherein said nucleic acid molecule comprises between 12 and 100 bases complementary to an RNA having K-Ras, H-Ras and/or N-Ras sequence.
67. The nucleic acid molecule of any of claims 53-59, wherein said nucleic acid molecule comprises between 14 and 24 bases complementary to an RNA having K-Ras, H-Ras and/or N-Ras sequence.
68. The nucleic acid molecule of any of claims 53-59, wherein said nucleic acid molecule is chemically synthesized.
69. The nucleic acid molecule of any of claims 53-59, wherein said nucleic acid molecule comprises at least one 2'-sugar modification.
70. The nucleic acid molecule of any of claims 53-59, wherein said nucleic acid molecule comprises at least one nucleic acid base modification.
71. The nucleic acid molecule of any of claims 53-59, wherein said enzymatic nucleic acid molecule comprises at least one phosphate backbone modification.

72. A mammalian cell comprising the nucleic acid molecule of any of claims 53-59.
73. The mammalian cell of claim 72, wherein said mammalian cell is a human cell.
74. A method of reducing K-Ras activity in a cell, comprising contacting said cell with the nucleic acid molecule of any of claims 53, 55, 57, 58 or 59, under conditions suitable for said reduction of K-Ras activity.
75. A method of reducing H-Ras activity in a cell, comprising contacting said cell with the nucleic acid molecule of any of claims 54, 56, 57, 58 or 59, under conditions suitable for said reduction of H-Ras activity.
76. A method of treatment of a subject having a condition associated with the level of K-Ras, comprising contacting cells of said subject with the nucleic acid molecule of any of claims 53, 55, 57, 58 or 59, under conditions suitable for said treatment.
77. A method of treatment of a subject having a condition associated with the level of H-Ras, comprising contacting cells of said subject with the nucleic acid molecule of any of claims 54, 56, 57, 58 or 59, under conditions suitable for said treatment.
78. The method of claim 76 further comprising the use of one or more drug therapies under conditions suitable for said treatment.
79. The method of claim 77 further comprising the use of one or more drug therapies under conditions suitable for said treatment.
80. A method of cleaving RNA having a K-Ras sequence comprising contacting an nucleic acid molecule of any of claims 53, 55, 57, 58 or 59, with said RNA under conditions suitable for the cleavage.
81. A method of cleaving RNA having a H-Ras sequence comprising contacting an nucleic acid molecule of any of claims 54, 56, 57, 58 or 59, with said RNA under conditions suitable for the cleavage.
82. The method of claim 80, wherein said cleavage is carried out in the presence of a divalent cation.

83. The method of claim 81, wherein said cleavage is carried out in the presence of a divalent cation.
84. The method of claim 82, wherein said divalent cation is Mg^{2+} .
85. The method of claim 83, wherein said divalent cation is Mg^{2+} .
86. The nucleic acid molecule of any of claims 53-59, wherein said nucleic acid molecule comprises a cap structure, wherein the cap structure is at the 5'-end, 3'-end, or both the 5'-end and the 3'-end of said nucleic acid molecule.
87. The nucleic acid molecule of claim 86, wherein the cap structure comprises a 3',3'-linked or 5',5'-linked deoxyabasic ribose derivative.
88. An expression vector comprising a nucleic acid sequence encoding at least one nucleic acid molecule of any of claims 53-59 in a manner that allows expression of the nucleic acid molecule.
89. A mammalian cell comprising an expression vector of claim 88.
90. The mammalian cell of claim 89, wherein said mammalian cell is a human cell.
91. The expression vector of claim 88, wherein said nucleic acid molecule is in a DNzyme configuration.
92. The expression vector of claim 88, wherein said expression vector further comprises a sequence for a nucleic acid molecule complementary to a nucleic acid molecule having a K-Ras sequence.
93. The expression vector of claim 88, wherein said expression vector further comprises a sequence for a nucleic acid molecule complementary to a nucleic acid molecule having a H-Ras sequence.
94. The expression vector of claim 88, wherein said expression vector comprises a nucleic acid sequence encoding two or more of said nucleic acid molecules, which may be the same or different.
95. The expression vector of claim 88, wherein said expression vector further comprises a sequence encoding an antisense nucleic acid molecule or siRNA

nucleic acid molecule complementary to a nucleic acid molecule having a K-Ras sequence.

96. The expression vector of claim 88, wherein said expression vector further comprises a sequence encoding an antisense nucleic acid molecule or siRNA nucleic acid molecule complementary to a nucleic acid molecule having a H-Ras sequence.
97. A method for the treatment of cancer comprising administering to a subject the nucleic acid molecule of any of claims 53-59 under conditions suitable for said treatment.
98. The method of claim 97, wherein said cancer is colorectal cancer.
99. The method of claim 97, wherein said cancer is lung cancer.
100. The method of claim 97, wherein said cancer is prostate cancer.
101. The method of claim 97, wherein said cancer is bladder cancer.
102. The method of claim 97, wherein said cancer is breast cancer.
103. The method of claim 97, wherein said cancer is pancreatic cancer.
104. The method of claim 97, wherein said method further comprises administering to said patient one or more other therapies under conditions suitable for said treatment.
105. The method of claim 78 wherein said other drug therapies are chosen from monoclonal antibody therapy, chemotherapy, radiation therapy, and analgesic therapy.
106. The method of claim 79 wherein said other drug therapies are chosen from monoclonal antibody therapy, chemotherapy, radiation therapy, and analgesic therapy.
107. The method of claim 104 wherein said other drug therapies are chosen from monoclonal antibody therapy, chemotherapy, radiation therapy, and analgesic therapy.

108. The method of claim 105, wherein said chemotherapy is selected from the group consisting of paclitaxel (Taxol), docetaxel, cisplatin, methotrexate, cyclophosphamide, doxorubin, fluorouracil carboplatin, edatrexate, gemcitabine, and vinorelbine.
109. The method of claim 106, wherein said chemotherapy is selected from the group consisting of paclitaxel (Taxol), docetaxel, cisplatin, methotrexate, cyclophosphamide, doxorubin, fluorouracil carboplatin, edatrexate, gemcitabine, and vinorelbine.
110. The method of claim 107, wherein said chemotherapy is selected from the group consisting of paclitaxel (Taxol), docetaxel, cisplatin, methotrexate, cyclophosphamide, doxorubin, fluorouracil carboplatin, edatrexate, gemcitabine, and vinorelbine.
111. A composition comprising a nucleic acid molecule of any of claims 53-59 and a pharmaceutically acceptable carrier.
112. A method of administering to a cell a nucleic acid molecule of any of claims 53-59 comprising contacting said cell with the enzymatic nucleic acid molecule under conditions suitable for said administration.
113. The method of claim 112, wherein said cell is a mammalian cell.
114. The method of claim 113, wherein said cell is a human cell.
115. The method of claim 112, wherein said administration is in the presence of a delivery reagent.
116. The method of claim 115, wherein said delivery reagent is a lipid.
117. The method of claim 116, wherein said lipid is a cationic lipid.
118. The method of claim 116, wherein said lipid is a phospholipid.
119. The method of claim 115, wherein said delivery reagent is a liposome.
120. A siRNA nucleic acid molecule which modulates expression of a nucleic acid molecule encoding HIV or a component of HIV.

121. An enzymatic nucleic acid molecule which modulates expression of a nucleic acid molecule encoding HIV or a component of HIV, wherein said enzymatic nucleic acid molecule is in an Inozyme, G-cleaver, Zinzyme or Amberzyme configuration.
122. An enzymatic nucleic acid molecule comprising a sequence selected from the group consisting of SEQ ID NOs. 6727-6799.
123. An enzymatic nucleic acid molecule comprising at least one binding arm wherein one or more of said binding arms comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6642-6726.
124. A siRNA nucleic acid molecule comprising a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6642-6726.
125. The nucleic acid of any of claims 120-124, wherein said nucleic acid molecule is adapted to HIV infection or acquired immunodeficiency syndrome (AIDS).
126. The enzymatic nucleic acid molecule of any of claims 121-123, wherein said enzymatic nucleic acid molecule has an endonuclease activity to cleave RNA having a HIV sequence.
127. The enzymatic nucleic acid molecule of claim 121, wherein said enzymatic nucleic acid molecule is in an Inozyme configuration.
128. The enzymatic nucleic acid molecule of claim 121, wherein said enzymatic nucleic acid molecule is in a Zinzyme configuration.
129. The enzymatic nucleic acid molecule of claim 121, wherein said enzymatic nucleic acid molecule is in a G-cleaver configuration.
130. The enzymatic nucleic acid molecule of claim 121, wherein said enzymatic nucleic acid molecule is in an Amberzyme configuration.
131. The enzymatic nucleic acid molecule of claim 123, wherein said enzymatic nucleic acid molecule is in a DNAzyme configuration.
132. The enzymatic nucleic acid molecule of claim 123, wherein said enzymatic nucleic acid molecule is in a Hammerhead configuration.

133. The enzymatic nucleic acid molecule of claim 127, wherein said Inozyme comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6648-6655.
134. The enzymatic nucleic acid molecule of claim 127, wherein said Inozyme comprises a sequence selected from the group consisting of SEQ ID NOs. 6733-6740.
135. The enzymatic nucleic acid molecule of claim 128, wherein said Zinzyme comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6656-6663 and 6723-6726.
136. The enzymatic nucleic acid molecule of claim 128, wherein said Zinzyme comprises a sequence selected from the group consisting of SEQ ID NOs. 6741-6748 and 6795-6799.
137. The enzymatic nucleic acid molecule of claim 130, wherein said Amberzyme comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6656-6688.
138. The enzymatic nucleic acid molecule of claim 130, wherein said Amberzyme comprises a sequence selected from the group consisting of SEQ ID NOs. 6762-6789.
139. The enzymatic nucleic acid molecule of claim 131, wherein said DNAzyme comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6656-6668 and 6718-6722.
140. The enzymatic nucleic acid molecule of claim 131, wherein said DNAzyme comprises a sequence selected from the group consisting of SEQ ID NOs. 6749-6761 and 6790-6794.
141. The enzymatic nucleic acid molecule of claim 132, wherein said Hammerhead comprises a sequence complementary to a sequence selected from the group consisting of SEQ ID NOs. 6642-6647.
142. The enzymatic nucleic acid molecule of claim 132, wherein said Hammerhead comprises a sequence selected from the group consisting of SEQ ID NOs 6727-6732.

143. The nucleic acid molecule of any of claims 120-124, wherein said nucleic acid molecule comprises between 12 and 100 bases complementary to a nucleic acid molecule encoding HIV.
144. The nucleic acid molecule of any of claims 120-124, wherein said nucleic acid molecule comprises between 14 and 24 bases complementary to a nucleic acid molecule encoding HIV.
145. The nucleic acid molecule of any of claims 120-124, wherein said nucleic acid molecule is chemically synthesized.
146. The nucleic acid molecule of any of claims 120-124, wherein said nucleic acid molecule comprises at least one 2'-sugar modification.
147. The nucleic acid molecule of any of claims 120-124, wherein said nucleic acid molecule comprises at least one nucleic acid base modification.
148. The nucleic acid molecule of any of claims 120-124, wherein said nucleic acid molecule comprises at least one phosphate backbone modification.
149. A mammalian cell comprising the nucleic acid molecule of any of claims 120-124
150. The mammalian cell of claim 149, wherein said mammalian cell is a human cell.
151. A method of reducing HIV activity in a cell, comprising contacting said cell with the nucleic acid molecule of any of claims 120-124, under conditions suitable for said reduction of HIV activity.
152. A method of treatment of a subject having a condition associated with the level of HIV, comprising contacting cells of said subject with the nucleic acid molecule of any of claims 120-124, under conditions suitable for said treatment.
153. The method of claim 151 further comprising the use of one or more drug therapies under conditions suitable for said treatment.
154. The method of claim 152 further comprising the use of one or more drug therapies under conditions suitable for said treatment.

155. A method of cleaving RNA of an HIV gene comprising contacting an enzymatic nucleic acid molecule of any of claims 121-123 with said RNA of a HIV gene under conditions suitable for the cleavage.
156. The method of claim 155, wherein said cleavage is carried out in the presence of a divalent cation.
157. The method of claim 156, wherein said divalent cation is Mg^{2+} .
158. The nucleic acid molecule of any of claims 120-124, wherein said nucleic acid molecule comprises a cap structure, wherein the cap structure is at the 5'-end, 3'-end, or both the 5'-end and the 3'-end of said nucleic acid molecule.
159. The nucleic acid molecule of claim 158, wherein the cap structure at the 5'-end, 3'-end, or both the 5'-end and the 3'-end comprises a 3',3'-linked or 5',5'-linked deoxyabasic ribose derivative.
160. An expression vector comprising a nucleic acid sequence encoding at least one nucleic acid molecule of any of claims 120-124 in a manner which allows expression of the nucleic acid molecule.
161. A mammalian cell comprising an expression vector of claim 160.
162. The mammalian cell of claim 161, wherein said mammalian cell is a human cell.
163. An expression vector comprising a nucleic acid sequence encoding at least one nucleic acid molecule of any of claims 122 or 123 in a manner which allows expression of the nucleic acid molecule, wherein said nucleic acid molecule is in a hammerhead configuration.
164. The expression vector of claim 160, wherein said expression vector further comprises a sequence for a nucleic acid molecule complementary to the RNA of HIV.
165. The expression vector of claim 160, wherein said expression vector comprises a nucleic acid sequence encoding two or more of said nucleic acid molecules, which may be the same or different.

166. The expression vector of claim 165, wherein said expression vector further comprises a sequence encoding a siRNA nucleic acid molecule complementary to the RNA of HIV gene.
167. A method for treatment of acquired immunodeficiency syndrome (AIDS) or an AIDS related condition comprising administering to a subject the nucleic acid molecule of any of claims 120-124 under conditions suitable for said treatment.
168. The method of claim 167, wherein said AIDS related condition is Kaposi's sarcoma, lymphoma, cervical cancer, squamous cell carcinoma, cardiac myopathy, rheumatic disease, or opportunistic infection.
169. The method of claim 167, wherein said method further comprises administering to said subject one or more other therapies.
170. The nucleic acid molecule of claim 121 or claim 123, wherein said nucleic acid molecule comprises at least five ribose residues, at least ten 2'-O-methyl modifications, and a 3'- end modification.
171. The nucleic acid molecule of claim 170, wherein said nucleic acid molecule further comprises phosphorothioate linkages on at least three of the 5' terminal nucleotides.
172. The nucleic acid molecule of claim 170, wherein said 3'- end modification is a 3'-3' inverted abasic moiety.
173. The method of claim 153 wherein said other drug therapies chosen from antiviral therapy, monoclonal antibody therapy, chemotherapy, radiation therapy, analgesic therapy, and anti-inflammatory therapy.
174. The method of claim 173, wherein said antiviral therapy is chosen from treatment with AZT, ddC, ddI, d4T, 3TC, Ribavirin, delvaridine, nevirapine, efavirenz, ritonavir, saquinavir, indinavir, amprenavir, nelfinavir, and lopinavir.
175. The method of claim 154 wherein said other drug therapies are chosen from antiviral therapy, monoclonal antibody therapy, chemotherapy, radiation therapy, analgesic therapy, and anti-inflammatory therapy.

176. The method of claim 175, wherein said antiviral therapy is chosen from treatment with AZT, ddC, ddI, d4T, 3TC, Ribavirin, delvaridine, nevirapine, efavirenz, ritonavir, saquinivir, indinavir, amprenivir, nelfinavir, and lopinavir.
177. The method of claim 169 wherein said other drug therapies are chosen from antiviral therapy, monoclonal antibody therapy, chemotherapy, radiation therapy, analgesic therapy, and anti-inflammatory therapy.
178. The method of claim 177, wherein said antiviral therapy is chosen from treatment with AZT, ddC, ddI, d4T, 3TC, Ribavirin, delvaridine, nevirapine, efavirenz, ritonavir, saquinivir, indinavir, amprenivir, nelfinavir, and lopinavir.
179. A pharmaceutical composition comprising a nucleic acid molecule of any of claims 120-124 in a pharmaceutically acceptable carrier.
180. The nucleic acid molecule of claim 120 or 121, wherein said component of HIV is nef.
181. The nucleic acid molecule of claim 120 or 121, wherein said component of HIV is vif.
182. The nucleic acid molecule of claim 120 or 121, wherein said component of HIV is tat.
183. The nucleic acid molecule of claim 120 or 121, wherein said component of HIV is rev.
184. The nucleic acid molecule of claim 120 or 121, wherein said component of HIV is LTR.
185. The nucleic acid molecule of claim 184, wherein said LTR is the 3'-LTR.
186. The nucleic acid molecule of claim 184, wherein said LTR is the 5'-LTR.
187. A method of administering to a cell a nucleic acid molecule of any of claims 120-124 comprising contacting said cell with the nucleic acid molecule under conditions suitable for said administration.
188. The method of claim 187, wherein said cell is a mammalian cell.

- 189. The method of claim 187, wherein said cell is a human cell.
- 190. The method of claim 187, wherein said administration is in the presence of a delivery reagent.
- 191. The method of claim 190, wherein said delivery reagent is a lipid.
- 192. The method of claim 191, wherein said lipid is a cationic lipid.
- 193. The method of claim 191, wherein said lipid is a phospholipid.
- 194. The method of claim 190, wherein said delivery reagent is a liposome.

**Figure 2: 2'-O-Me substituted Amberzyme
Enzymatic Nucleic Acid Motif**

$$\overline{U}, \overline{C} = 2'-NH_2-U, C$$

Lower case = 2'-O-Me

Uppercase = Ribo

Amberzyme

(SEQ ID NO. 6806)

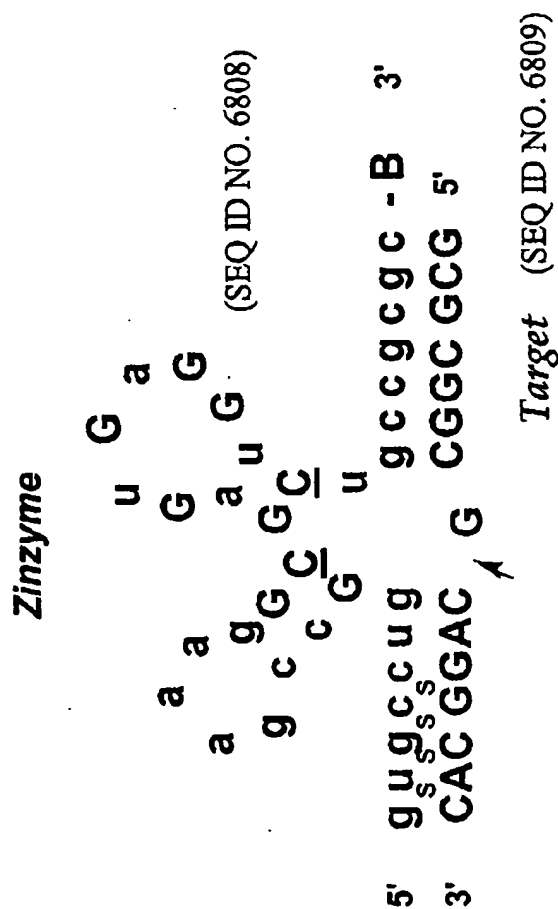
48

Cleavage site

Substrate

(SEQ ID NO. 6807)

Figure 3: Stabilized Zinzyme Ribozyme Motif



Legend

Uppercase: indicates natural ribo residues

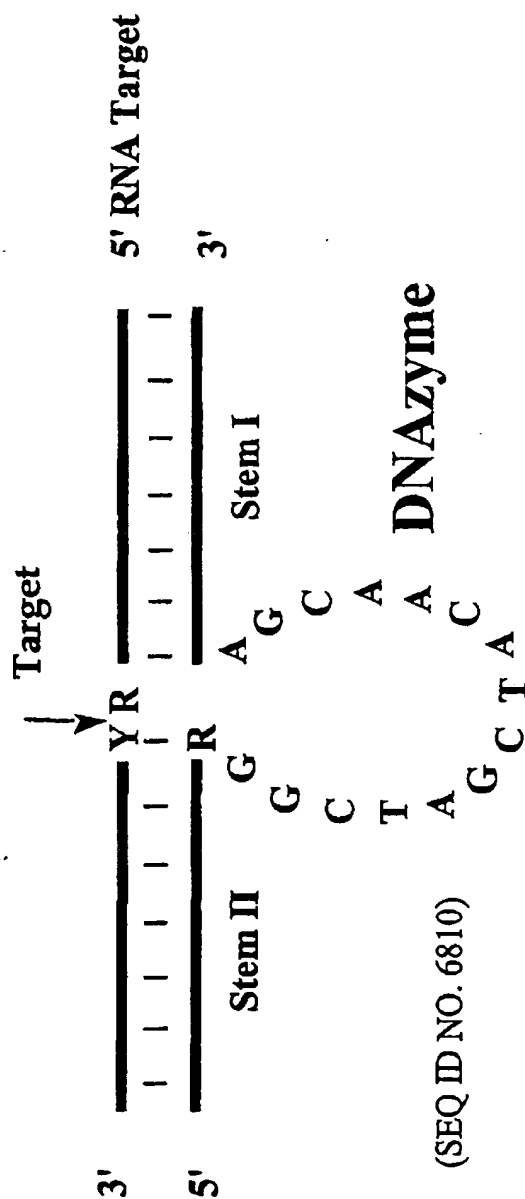
C : indicates 2'-deoxy-2'-amino Cytidine

Lowercase: 2'-O-methyl

S: phosphorothioate/phosphorodithioate linkage

B: 3'-3' abasic moiety

Figure 4: DNAzyme Motif



Legend

Y = U or C
R = A or G

